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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07K 13/00, C07H 17/00, C12P 21/06, C12N 15/00, 1/20, G01N 33/566, 33/563, A61K 37/00

A1

(11) International Publication Number: **WO 94/24158**

(43) International Publication Date: 27 October 1994 (27.10.94)

(21) International Application Number: PCT/US94/04132

(22) International Filing Date: 14 April 1994 (14.04.94)

(30) Priority Data:
048,164 14 April 1993 (14.04.93) US

(60) Parent Application or Grant
(63) Related by Continuation
US 048,164 (CIP)
Filed on 14 April 1993 (14.04.93)

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(81) Designated States: AU, BB, BG, BR, BY, CA, CN, CZ, FI, GE, HU, JP, KG, KR, KZ, LK, LV, MD, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SI, SK, TJ, UA, US, UZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: CELLULOSE BINDING DOMAIN

(57) Abstract

A cellulose binding domain (CBD) having a high affinity for crystalline cellulose and chitin is disclosed, along with methods for the molecular cloning and recombinant production thereof. Fusion products comprising the CBD and a second protein are likewise described. A wide range of applications are contemplated for both the CBD and the fusion products, including drug delivery, affinity separations, and diagnostic techniques.

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CELLULOSE BINDING DOMAIN

This application is a Continuation-In-Part of United States Application Serial No. 08/048,164, filed April 14, 1993.

1. INTRODUCTION

The present invention relates to a cellulose binding domain (CBD) that binds to water-insoluble forms of cellulose and chitin, including crystalline forms, with a remarkably high affinity and in a reversible manner. The CBD of the present invention, which has been isolated and which is substantially free of other proteins with which the CBD is naturally associated, finds use, for example, in the bio-immobilization of a wide variety of substances, especially biologically active molecules, to cellulose. Fusion products comprising the CBD and a second protein of interest are also disclosed, including applications in methods for their preparation. Such fusion proteins enjoy a wide range of useful applications, including applications in separation, purification and diagnostic methods.

2. BACKGROUND OF THE INVENTION

2.1. Immobilization of Proteins

Immobilization of chemical substances, including biologically active proteins, is of great importance to industry. Various methods of immobilization have been developed in recent years. However, most of these methods require chemical modification of a solid matrix. Typically, these modifications require the covalent attachment of a ligand to the matrix resulting, in many cases, in loss

of activity of the ligand as well as the inclusion of toxic organic compounds that must be removed before the matrix can be used in medicine or food processing. A typical example of a widely used product is Protein A-Sepharose. This highly expensive product is used for the purification of IgG by affinity chromatography, as well as for many diagnostic protocols.

The use of chimeric proteins, *i.e.*, those that contain a functional domain (catalytic or otherwise) together with a binding domain, is relatively new but has already proven to be very useful, especially in protein purification methods. For example, the glutathione S-transferase gene fusion system is designed to express a gene of interest fused to the C-terminal of glutathione S-transferase. The recombinant protein is purified by affinity chromatography using glutathione-Sepharose column.

Another example of a chimeric protein having a functional domain and a binding domain is the Protein-A gene fusion vector which has been designed to permit a high level of expression of fusion proteins in both *E. coli* and *Staphylococcus aureus* cells. B. Nilsson, *et al.* (1985) EMBO J. 4(4):1075-1080. The IgG binding domain of Protein A provides a rapid purification method of the fusion protein using IgG-Sepharose columns. Similar systems have been developed based on beta-galactosidase fusion proteins purified on IPTG-Sepharose or metal chelate chromatography or histidine hexamer fusion proteins using Ni-resin columns. All these methods use expensive matrices such as Sepharose, acrylic beads, or glass beads that require costly chemical modifications and in many cases, the use of highly toxic compounds. Cellulose, on the other hand, has

excellent physical properties and is inexpensive, thus providing an attractive solid matrix for protein immobilization.

2.2. Cellulose

Cellulose has been very useful for immobilization of endo-beta-glucosidase, an enzyme that is used for wine and fruit juice treatments (Shoseyov *et al.* J. Agric. Food Chem. 38:1387-1390 [1990].). However, immobilization requires the chemical modification of the cellulose and results in a fluffy compressible material that is not suitable for applications involving packed columns.

A cellulose substrate, to which a ligand could be bound without resort to chemical modification (*e.g.*, "bioimmobilized"), would possess the advantage that the resulting solid matrix is natural and non-toxic (having required no "dirty chemical modifications"). It would be further advantageous if the resulting solid matrix retained its physical properties, as well as its relatively low price. At present, cellulose prices are 100-500 fold lower than those of glutathione-Sepharose and IPTG-Sepharose, making cellulose an attractive, inexpensive matrix that can be used safely in food and pharmaceutical industries. Recently, Greenwood et. al. (FEBS Lett. 224(1): 127-131 [1989].) fused the cellulose binding region of *Cellulomonas fimi* endoglucanase to the enzyme alkaline phosphatase. The recombinant fusion protein retained both its phosphatase activity and the ability to bind to cellulose. *See*, also U.S. Patent No. 5,137,819 granted to Kilburn *et al.*, incorporated by reference herein.

Unfortunately, the *Cellulomonas fimi* cellulose binding region exhibits a relatively low

affinity to cellulose. For instance, more than 30% of the fusion protein is washed off by 50 mM Tris-HCl (pH 7.5) in 0.5 M NaCl. A second disadvantage of the *C. fimi* cellulose binding region is that the cellulose fibers are disrupted upon binding to the *C. fimi* binding region (Din *et al.*, Bio/Technology 9:1096-1098 [1991]). Therefore, even though the *C. fimi* cellulose binding region may not exhibit direct cellulase activity, this disruption of the fibers of the cellulose substrate, which is tantamount to a physical change in the morphology of the solid matrix, is equally problematic and undesirable. (*see*, further, the discussion in Section 7.3, below.)

2.3. Cellulase

Many cellulases and other hydrolytic enzymes, such as chitinases, have high affinities for their substrates (Shoseyov, *et al.*, PNAS USA 87:2192-2195 [1990]; Cabib, Methods Enzymol. 161, pp. 460-462 [1988]). It has previously been shown that strong binding between crystalline cellulose and the cellulase is directly related to that cellulase's ability to degrade crystalline cellulose (Klyosov, Biochemistry 29:10577-10585 [1990]), whereas strong binding is not necessary for that cellulase's ability to degrade amorphous cellulose.

Shoseyov, *et al.*, (PNAS USA 87:2192-2195 [1990]) report purification of the cellulase "complex" from *Clostridium cellulovorans*. This cellulase "complex" exhibits cellulase activity against crystalline cellulose, as well as carboxymethylcellulose, and is a large protein complex consisting of several different polypeptides. It has been found that a large (ca. 200 kD) major cellulose binding protein (CbpA), with no apparent enzyme

activity, must participate with the catalytic enzyme in order for the catalytic enzyme to breakdown crystalline cellulose. However, no such participation by the CbpA is necessary for the enzymatic degradation of amorphous cellulose. Shoseyov, *et al.*, (PNAS USA 89:3483-3487 [1992]) report the cloning and DNA sequencing of the gene for CbpA, including a description of four separate "putative" cellulose binding domains within the CbpA.

2.4. Heat Shock Protein (HSP)

Heat shock proteins (HSP) are induced in prokaryotic and Eukaryotic species under various conditions of stress. The HSP's are grouped into families of homologous proteins based on their molecular masses.

The 60 kD HSP (hsp 60) family, which retained a uniquely high level of sequence conservation during evaluation is a focus of interest as a potential antigen in autoimmune diseases. W. Van Eden (1991) Immunol. Rev. 121:1; W. Van Eden, Thole R. Van der Zee, A. Noordzij, J.D.A. Van Einbden, E.J. Hensen, and I.R. Cohen (1988) Nature 331:171; M.B.J. Billingham S. Carney, R. Bulter, and M.J. Colston (1990) J. Exp. Med. 171:339.

There is experimental evidence that response to hsp 60 is subject to regulatory T cell control. It has been suggested that T cell reactivity is directed, at least partly, against the mammalian endogenous HSP. B. Herman *et al.* (1991) Eur. J. Immunol. 21:2139.

The genes coding for hsp 60 protein family have been cloned and sequenced from a number of different species including *Mycobacterium tuberculosis, Microbacterium leprae, Mycobacterium bovis BCG, E. coli*, chinese hamster rat mouse and

human cells. V. Mera *et al.* (1986) Proc. Nat. Acad. Sci. USA 83:7013; S. Jindal *et al.* (1989) Mol. Cell. Biol. 9:2279; O.J. Picketts *et al.* (1989) J. Biol. Chem. 264:12001; T.M. Shinnick (1987) J. Bacteriol. 169:1080; T.M. Shinnick *et al.* (1988) Infect. Immun. 56:446; T.J. Venner and R.S. Gupta (1990) Nucl. Acids. Res. 18:5309.

As described *infra*, according to the present invention, the heat shock protein (HSP), portions thereof, anti-HSP antibodies or portions thereof, can be used in CBD-fusion products.

Immune response to Mycobacterial and human hsp 60 has been implicated in the development of autoimmune diabetes in human and animal models.

Besides being an immunodominant antigen, the hsp 60 family of proteins in various systems has been shown to perform a "molecular chaperone" role in the proper folding of newly synthesized polypeptide chains and, in some cases, their assembly into oligomeric protein complexes. S.M. Hemmingsen *et al.* Nature 333:330; R.J. Ellis (1990) Sem. Cell Biol. 1:1-9.

In a model system in mice for insulin dependent diabetes mellitus (IDDM) - T lymphocytes responding to the hsp 60 antigen are detectable at the onset of insulitis and it is likely that these T lymphocytes can also recognize the Beta cell hsp 65 cross reactive antigen. D. Elias *et al.* (1990) Proc. Nat. Acad. Sci. USA 87:1576-1580.

Thus, there remains an unfulfilled need to discover a cellulose binding protein that exhibits a high but reversible affinity for cellulose, particularly crystalline cellulose, but which manifests neither cellulase activity nor an ability to disrupt the fibers of the crystalline cellulose

substrate (*i.e.*, does not exhibit an "amorphogenic" effect).

3. SUMMARY OF THE INVENTION

The inventors describe herein the identification, molecular cloning and cellulose binding characteristics of a novel cellulose binding domain (CBD) protein. Also, disclosed is the construction of an expression system for the production of a fusion product comprising the CBD and a second protein of interest.

The invention relates to the discovery that the CBD is able to function independently of the other proteins or polypeptides with which it is naturally associated. Moreover, it has been discovered unexpectedly that the CBD has a high affinity for crystalline cellulose and chitin, having a K_d ranging from about 1.5μM to about 0.5μM, preferably ranging from about 1.4μM to about 0.8μM. In particular, with various samples of crystalline cellulose, the CBD exhibits a K_d of about 1.2μM or less. The CBD can be further characterized in that it possesses virtually no cellulase activity and, quite surprisingly, the CBD exhibits no morphology-altering characteristics (*i.e.*, no amorphogenic effects). It has also been discovered that CBD-fusion products comprising CBD and a second protein retain the avid binding capacity of the CBD to cellulose.

The invention is also related to the discovery that the CBD demonstrates absolute binding to cellulose over a wide range of pH and under different buffering conditions, that large quantities of CBD bind to crystalline cellulose, and that exposure to water fails to release CBD from cellulose. In stark contrast, the major CbpA protein, as well as

the binding region from *C. fimi*, are readily dissociated from cellulose on exposure to water. Indeed, exposure to denaturing solutions, such as 6 M guanidine-HCl, 6 M urea or nonionic surfactants, is required to release the CBD from cellulose. Thus, the CBD protein functions and behaves quite independently of the rest of the other proteins with which it is naturally associated in its binding to cellulose.

Thus, the present invention provides an isolated CBD protein capable of binding cellulose with high affinity and which is substantially free of other proteins with which it is naturally associated.

In one embodiment of the present invention, a CBD protein comprises the amino acid sequence shown in Figure 1. In another embodiment of the present invention, the CBD protein comprises an amino acid sequence having at least 70% homology, preferably 80% homology, to the amino acid sequence disclosed in Figure 1. In another aspect of the present invention, a nucleic acid is contemplated having at least 60% homology, preferably 70-80% homology, to the nucleic acid sequence depicted in Figure 1.

In yet another embodiment of the present invention, the CBD protein has a high binding affinity to cellulose. More preferably, the CBD of the present invention has a K_d of about 1.5μM or less, most preferably 1.0μM or less.

In yet another embodiment, the present invention provides a CBD fusion protein comprised of a CBD protein capable of binding cellulose with high affinity and a second protein wherein, when said CBD protein is one which occurs in nature, said CBD protein is substantially free of other proteins with which it is naturally associated. In a particular embodiment of the present invention, the second

protein is Protein A. In another embodiment of the present invention, the second protein is an HSP protein. In yet another embodiment of the present invention, the second protein fused to the CBD may be comprised of two or more polypeptide regions. For example, the CBD may be fused to the variable light chain (V_L) and the variable heavy chain (V_H) of an antibody or functional portions thereof.

A further embodiment of the present invention provides a method for the production of a CBD or a CBD fusion product. An exemplary procedure, which can be applied either to CBD alone or to a CBD fusion product, but which is recited herein for a CBD fusion product, may comprise the following steps: providing nucleic acid encoding the CBD fusion product wherein said CBD fusion product is comprised of a CBD and a second protein, said CBD being capable of binding cellulose or chitin with high affinity and being substantially free of other proteins with which it is naturally associated; transfecting a host cell with the nucleic acid or using an equivalent means for introducing the nucleic acid into the host cell; and (2) culturing the transformed host cell under conditions suitable for expression of the CBD fusion protein. In the above method, the second protein may be further comprised of an N-terminal amino acid and a C-terminal amino acid. Transfection of the host cell can be effected in a number of ways well known to those of ordinary skill in the art, including, but not limited to, electroporation, injection, calcium chloride precipitation and retroviral introduction. Furthermore, the nucleic acid can be either integrated with the genome of the host cell or not.

In a further aspect of the present invention, a method of the purification of a CBD

fusion product is provided comprising contacting a mixture comprising a recombinant CBD fusion product with an effective amount of cellulose under conditions suitable for the formation of an insoluble binding complex comprising cellulose and the recombinant fusion product; isolating the insoluble cellulose-CBD fusion product binding complex from the mixtures; and recovering the CBD fusion protein from the cellulose-CBD fusion product binding complex.

In a particular embodiment of the present invention, the method of purifying a CBD fusion protein of the present invention further comprises providing nucleic acid encoding a cleavage site upstream of the N-terminal amino acid of the second protein of the CBD fusion product.

In another embodiment of the present invention, the method for purifying a CBD fusion protein further comprises providing nucleic acid encoding a cleavage site downstream of the C-terminal amino acid of the second protein of the CBD fusion protein.

In yet another embodiment of the present invention the method for purifying a CBD fusion protein of the present invention further comprises providing nucleic acid encoding a first cleavage site upstream of the N-terminal amino acid of the second protein and a second cleavage site downstream of the C-terminal amino acid of the second protein of the CBD fusion protein.

Similarly, CBD is purified by contacting a mixture comprising CBD with an effective amount of cellulose. Isolation of the resulting insoluble CBD-cellulose binding complex followed by treatment of the binding complex with a releasing reagent provides the purified CBD.

Another aspect of the present invention provides an isolated nucleic acid encoding a CBD protein which is capable of binding cellulose with high affinity and which is substantially free of other nucleic acid with which it is naturally associated. An isolated nucleic acid encoding a protein of the present invention may be useful as a probe in screening cDNA or genomic libraries for sequences having homology to the *cbd* gene.

In further embodiments, the present invention provides for a host cell comprised of nucleic acid encoding a CBD of the present invention.

An additional aspect of the present invention relates to a diagnostic kit for the detection of a substance of interest comprising: (a) a CBD fusion product comprising (i) a CBD capable of binding to cellulose with high affinity and substantially free of other proteins with which it is naturally associated, and (ii) a second protein capable of binding a substance of interest; (b) a detectable label; and (c) cellulose. In such a diagnostic kit, the cellulose can be replaced by chitin. In specific embodiments of the present invention, the second protein of the CBD fusion product can be Protein A, HSP protein, HSP antibody, HSP-related protein, peptide or antigenic portion thereof. The term "peptide" is meant to include molecules comprising 2-20 amino acids. The CBD fusion product of the disclosed diagnostic kit may further comprise a ligand affinity bound to the second protein, such ligand capable of binding a substance of interest. By "ligand" is meant any molecule that is able to bind a second molecule by any non-covalent means. For example, a primary IgG can be affinity bound to Protein A fused to CBD. The IgG may then

serve as a ligand for a particular protein, peptide or hormone.

In another aspect of the present invention, an immunoassay method of detecting the presence of a substance of interest in a test sample is disclosed comprising: (a) incubating a test sample, which may contain a substance of interest, with a sufficient amount of a CBD fusion product comprising (i) a CBD capable of binding to cellulose with high affinity and substantially free of other proteins with which it is naturally associated, and (ii) a second protein capable of binding the substance of interest, under conditions that allow for the binding of the substance of interest to the second protein of the CBD fusion product; (b) adding an amount of cellulose effective to bind the amount of the CBD fusion product used in step (a) to provide an insoluble cellulose-CBD fusion product binding complex; (c) separating the insoluble cellulose-CBD fusion product binding complex from unbound components; (d) incubating the insoluble cellulose-CBD fusion product binding complex with a sufficient amount of a detectable label, the label capable of binding to the substance of interest; and (e) separating the insoluble cellulose-CBD fusion product binding complex of step (d) from unbound components and determining the presence or absence of the label, to provide an indication of the presence or absence of the substance of interest in the test sample.

Another method is also disclosed for the detection of a substance of interest in a test sample comprising: (a) contacting a test sample, which may contain a substance of interest, with an insoluble matrix capable of immobilizing the substance of interest; (b) incubating the insoluble matrix with a

sufficient amount of a CBD fusion product comprising (i) a CBD capable of binding to cellulose with high affinity and substantially free of other proteins with which it is naturally associated, and (ii) a second protein capable of binding the immobilized substance of interest, under conditions that allow for the binding of the immobilized substance of interest to the second protein of the CBD fusion product; (c) separating the insoluble matrix of step (b) from unbound components; (d) incubating the insoluble matrix of step (c) with a detectable label capable of binding the substance of interest or the CBD fusion product under conditions that allow for the binding of the label to the substance of interest or the CBD fusion product; and (e) separating the insoluble matrix of step (d) from unbound components and determining the presence or absence of the label, to provide an indication of the presence or absence of the substance of interest in the test sample.

This method may further comprise (i) contacting the insoluble matrix of step (c) with a sufficient amount of cellulose under conditions that allow for the binding of the cellulose to the CBD fusion product to form a cellulose-CBD fusion product binding complex, and (ii) separating the insoluble matrix of step (i) from unbound components, including unbound cellulose. This method can use a label that is capable of binding the substance of interest or the cellulose-CBD fusion protein binding complex, in particular, the cellulose of the cellulose-CBD fusion protein binding complex. The test sample may be a bodily fluid, including, but not limited to, blood, urine, semen, saliva, mucus, tears, vaginal secretions, and the like. As usual, the cellulose can

be replaced by chitin. Also, the insoluble matrix may be an electrophoresis gel blot.

In a specific embodiment of the present invention, the method is designed for the detection of a protein or peptide; thus, the second protein of the CBD fusion product may be an antibody against the protein or peptide. The antibody may be a monoclonal antibody or a polyclonal antibody. Alternatively, the CBD of the present invention may be conjugated to the antibody of interest either directly or through a linker moiety.

The substance of interest may also comprise a biotinylated probe bound to a protein, peptide, hormone, nucleic acid or other probe-targetable molecule. In this case, the preferred second protein is streptavidin. Where the label includes an enzyme, the method further comprises adding a sufficient amount of a substrate for the enzyme, which substrate is converted by the enzyme to a detectable compound.

The assay may also be carried out in a competitive mode. Hence, the above-described method may be modified such that step (d) is performed by incubating the insoluble cellulose-CBD fusion product binding complex with a sufficient amount of a detectable label comprising a labeled substance of interest, the label capable of binding to any second protein of the CBD fusion product which remains unbound to the substance of interest, and in which step (e) is performed by separating the insoluble cellulose-CBD fusion product binding complex of step (d) from unbound components and comparing the signal observed from the test sample relative to the signal observed from a control sample.

In a preferred embodiment of the present invention, the CBD fusion product is included in a dip

stick. Hence, it is also an object of the present invention to provide a dip stick useful in detecting a substance of interest in a test sample comprising a CBD fusion product, the CBD fusion product comprising (i) a CBD capable of binding to cellulose with high affinity and substantially free of other proteins with which it is naturally associated, and (ii) a second protein capable of binding a substance of interest. Preferably, the second protein is selected from the group consisting of Protein A, HSP protein, HSP antibody, cross-reactive HSP-related protein or peptide or an antigenic portion thereof, an enzyme, hormone, antigen, and antibody.

Likewise, it is also an object of the present invention to provide a signal amplification system comprising: (a) a first CBD fusion product comprising (i) a CBD capable of binding to cellulose with high affinity and substantially free of other proteins with which it is naturally associated, and (ii) a second protein capable of binding a chimeric probe, the probe further capable of binding a substance of interest; and (b) a second CBD fusion product comprising (i) a CBD capable of binding to cellulose with high affinity and substantially free of other proteins with which it is naturally associated, and (ii) an enzyme capable of acting on a substrate to produce a detectable compound.

In another embodiment, a signal amplification system is provided which comprises: (a) a CBD fusion product comprising (i) a CBD capable of binding to cellulose with high affinity and substantially free of other proteins with which it is naturally associated, and (ii) a second protein capable of binding a chimeric probe, the probe further capable of binding a substance of interest; and (b)

labeled CBD, the CBD retaining its capacity to bind to cellulose with high affinity and substantially free of other proteins with which it is naturally associated.

These signal amplification systems may include the chimeric probe and may further include a cellulose matrix, preferably a pebble-milled cellulose.

These signal amplification systems may include systems designed to identify nucleic acid from cells or tissue and systems designed to extend the length of identified nucleic acid in either direction which employ oligonucleotide-directed thermocyclic DNA amplification of the missing sequences, wherein a combination of a DNA-specific primer and a degenerate, vector-specific, or oligo-dT-binding second oligonucleotide can be used to prime specific synthesis.

Finally, it is a further object of the present invention to provide a drug delivery system comprising CBD associated with a drug, the CBD retaining its capacity to bind to cellulose with high affinity and substantially free of other proteins with which it is naturally associated. In such a drug delivery system, the drug is conjugated to the CBD either directly or through a linker moiety. Many methods of conjugation exist and are known in the art. For example, acyl activation agents exist, such as cyclohexylcarbodiimide, which can be used to form amide or ester bonds. Thus, a drug having a nucleophilic group, such as amino or hydroxy may be attached to the carboxy terminal end of CBD. In one embodiment such a drug delivery system can be a slow or sustained drug delivery system wherein the drug of interest is slowly released from the CBD bound to cellulose.

In one embodiment, the drug to be delivered is an antifungal agent. Preferred agents, include, but are not limited to, Amphotericin B, Nystatin, and Undecylenic Acid. The drug may generally be an imidazole, such as Clotrimazole. It is contemplated that such a drug delivery system can be incorporated into a composition that can be administered parenterally, orally, topically or by inhalation. In particular, routes of administration include, but are not limited to, intranasal, opthalmic or intravaginal. Furthermore, the composition may be in the form of a solid, gel, liquid or aerosol.

The drug delivery system described herein are useful for the delivery of a drug to an infectious or disease-causing agent, such as a yeast or fungal agent whose cellular membrane contains a cellulosic or chitinic substance. Examples of such agents, include, but are not limited to, *Aspergillus fumigatus*, a member of the genus *Candida* or *Monilia*, or an epidermatocyte. By employing the drug delivery system of the present invention, it is anticipated that the dosages required for effective treatment of the disease or infection will be much reduced, thus, improving the effectiveness of the antifungal or antimycosal drugs, which are typically also quite toxic. It is hoped that side effects are, thus, also minimized or, even better, eliminated.

Yet another aspect of the present invention relates to the ability of CBD to modify the growth of plant tissues. Such ability finds application in the agricultural industry, for example, where it may be desirable to promote pollen tube growth and/or root growth. In addition, since CBD in high concentration has the ability to inhibit root growth it is useful to prevent the devlopment of undesirable plants, for

example, "weed" plants. In another example, the CBD finds application in enhancing growth of fungi, such as mushrooms, wherein the CBD binds chitin and stimulates the growth and/or development of developing basidiocarps or fruiting bodies.

Yet another aspect of the present invention relates to the ability of CBD to bind to chitin of insect exoskeletons and other insect parts, including the mid-gut. Such ability finds application in the use of CBD as a bio-pesticide wherein the CBD is linked to a microorganism useful for controlling insect pests, such as *Bacillus thuringiensis* (BT) or other microorganism expressing the BT toxin gene, or such as a chitinase secreting microorganism or fungus. The CBD functions to bind the microorganism to the chitinous portions of the insect where the microbially produced toxin or enzyme leads to death of the insect pest. Microorganisms which display chitinase activity include bacterial strains from the genera Enterobacter and Streptomyces, and fungal strains from the genera Aspergillus, Penicillium and Trichoderma, etc. In one mode of this embodiment of the invention, the CBD-microorganism complex can be applied directly to plant parts suspected or at risk of infestation by insect pests or, alternatively, the CBD-microorganism complex can be immobilized on bait stations which are set out in the infested fields or on plants infested with insect pests. In another alternative mode of this embodiment of the invention, a CBD-microorganism complex is applied to wood or cellulose-based products to prevent or control infestation by wood destroying insect pests. For example, a CBD-microorganism complex is applied to telephone poles or wood or cellulose-based construction components where the CBD component binds tightly to invading insects and the

microorganism of the complex is effective to control the insect pests.

Still another aspect of the present invention relates to the ability of CBD to bind to chitin found in fungi, including fungi which are useful for bioremediation or degradation of toxic environmental pollutants. According to one mode of this embodiment, a CBD fusion protein is used in which the second component is an enzyme which degrades the environmental pollutant. The CBD-enzyme fusion protein can be immobilized on a cellulose substrate and the device used to remove the pollutant from the environment by enzymatic degradation, for example, by placing the device in a stream of surface or underground water. According to an alternative mode of this embodiment of the invention, the CBD is bound to a fungus which secretes an enzyme which is useful for biomediation, *i.e.*, which degrades an environmental pollutant such as a pesticide. The CBD-fungus complex is then immobilized on a cellulose substrate and provides a useful device to enzymatically degrade an environmental pollutant such as a pesticide including DDT or such as wood containing products left over from logging or saw milling operations. In one illustrative example, CBD is complexed with a white rot fungus, for example *Phanerochaete chrysosporium*, which produces lignin peroxidase, manganese peroxidase and degrades the pesticide DDT. The CBD-fungus complex is useful for bioremediation, for example, to remove a pesticide such as DDT from the environment.

4. DESCRIPTION OF THE FIGURES

Figure 1. Nucleotide (top, orig.; second line, complement) and deduced amino acid sequence of CBD.

Figure 2. Preparation and cloning of the gene fragment encoding for CBD.

A. Analysis of the primary structure of CbpA, which contains an N-terminus signal peptide, unique CBD region, 4 hydrophilic repeats (white arrows), and 9 hydrophobic repeats (black arrows).

B. PCR primer placement along the *cbp*A gene. Included for clarity are the primer sequences and the *cbp*A DNA sequence of the CBD flanking regions. The PCR product contains *Nco*I and *Bam*HI sites, underlined. Also note that the ATG start codon for the gene fragment is located within the *Nco*I site, and the TAG stop codon is adjacent to the *Bam*HI site.

C. Schematic of pET-CBD, containing the CBD gene fragment cloned into the pET-8c vector. The vector contains the necessary transcriptional and translational signals for inducible CBD production.

Figure 3. Expression and purification of the CBD protein.

Whole cell proteins from cells harboring pET-8c (*lane 2*), whole cell proteins from cells harboring pET-CBD (*lane 3*), cytosolic fraction from lysed pET-CBD cells (*lane 4*), Guanidine HCl-solubilized membrane/ inclusion body fraction from lysed pET-CBD cells (*lane 5*), final PC buffer wash of Avicel® pellet (*lane 6*), and purified CBD protein (*lane 7*) were loaded on a 15% acrylamide gel. Each lane was loaded with 0.005% of the total protein of each fraction, except lane 6 which is a 10-fold concentrate. Prestained molecular mass markers (*lanes 1,8*) have mobilities of approximately 2.6, 5, 12.7, 18.1, 29, and 44 kDa.

Figure 4. Time course of CBD-Avicel® binding. CBD (2.0 μmol total protein) and Avicel® (1 mg/ml) were equilibrated as described in Section 7, below, except that a larger total volume was used to provide samples taken at various time points. Each time point sample was washed and assayed as described in Section 7. *see*, also, Table I of Figure 9, below.

Figure 5. Double reciprocal plot of CBD binding to Avicel.® 0.5 mg Avicel® is represented by closed squares (B), 1 mg Avicel® by closed circles (J), and 2 mg by closed triangles (H). Inset: PC_{max} versus the amount of Avicel® used. The assay volume was 1.0 ml.

Figure 6. Scatchard Plot CBD binding to Avicel®. The [PC]/[P] vs. PC for 3 amounts of Avicel® are shown. The [PC]/[P] is expressed as a dimensionless ratio, and the [PC] is shown in μM. 1 mg Avicel® is represented by closed circles, 2 mg Avicel® by open circles, and 3 mg by closed squares.

Figure 7. Double reciprocal plot CBD binding to Cellulon®. The incubation mixture contained 0.5 mg Cellulon® per ml.

Figure 8. CBD-ProtA fusion protein binds both Cellulose and IgG. 1M Acetic acid selectively releases CBD-ProtA:IgG "bond", but not the CBD-ProtA:cellulose "bond".

Figure 9. Adsorption of CBD protein to insoluble substrates.

Figure 10. Overexpression and purification of CBD.

Figure 11. Schematic of a signal amplification system in which labeled CBD detects cellulose or chitin to which is bound a CBD fusion product. The substance of interest is bound, in turn, to a chimeric probe.

Figure 12. Schematic of a signal amplification system in which the substance of interest is bound to a ternary CBD fusion product: CBD-ProteinA(IgG).

Figure 13. IgG purification by CBD-ProtA-Cellulose as demonstrated by detection of samples on gel electrophoresis. Lane a is human serum. Lane b are human serum proteins that did not bind to CBD-ProtA-Cellulose. Lane c is the first PBS wash of samples, after binding of the IgG to the ProtA has taken place, showing unbound protein. Lane d is the IgG fraction recovered by acetic acid wash.

Figures 14A and 14B. Demonstration that CBD-treated cellulose fibers remain intact, *i.e.*, CBD has no cellulose disruption amorphogenesis activities. Figure 14 A shows a cotton fiber treated with CBD. Figure 14 B shows untreated cotton fibers (control).

Figure 15. Demonstration of the effect of pH and NaCl concentrations on the binding capacity of CBD-ProtA to cellulose.

Figure 16. Demonstration of CBD binding to *Aspergillus niger*. Lane a is *Aspergillus niger* with CBD. Lane b is *Aspergillus niger* without CBD (control). Lane c is CBD alone.

Figure 17. Demonstration of CBD binding to *Spodoptera littoralis*. Lane a is the mid-gut membrane of *Spodoptera littoralis* without CBD (control). Lane b is the mid-gut membrane of *Spodoptera littoralis* with CBD.

Figure 18. Demonstration of CBD binding to *Heliothis armigera*. Lane a is mid-gut membrane of *Heliothis armigera* without CBD (control). Lane b is mid-gut membrane of *Heliothis armigera* with CBD.

Figure 19. Demonstration of the effect of CBD and BSA (as a control) on pollen tube growth.

Figures 20A and 20B. Demonstration of the effect of CBD on crystalline cell wall components of pollen tubes that were stained with white fluorescent brightener (calcofluor). Figure 20A shows that CBD treated pollen tubes produced non-crystalline pollen tube tip as indicated by the absence of the bright color at the tip zone. Figure 20B shows that a control pollen tube tip contains more crystalline cell wall components as indicated by the bright color.

Figures 21A and 21B. Demonstration of gold-immunolabeling of CBD treated pollen tubes. Figure 21A demonstrates that in pollen treated with CBD, gold-labeling is along the pollen tube but preferably at the tip zone, as indicated by the intensive dark dots at the tip zone. Figure 21B shows the control, *i.e.*, pollen without the CBD treatment.

Figure 22. Demonstration of the effect of CBD on root growth of *Arabidopsis thaliana* seedlings. Different letters indicate statistically significant differences between the root length values ($p \leq 0.05$).

Figure 23. Demonstration of gold-immunolabeling of CBD treated *Arabidopsis thaliana* seedlings. This figure shows that the gold-labeling is restricted to the root and no labeling is shown on the shoot.

Figure 24. Demonstration of *Arabidopsis thaliana* seedling staining with white fluorescent brightener (calcofluor). This figure demonstrates that only the root is stained indicating accessible cellulose.

5. DETAILED DESCRIPTION OF THE INVENTION AND DEFINITIONS

The present invention is directed to the identification of cellulose binding domain (CBD) protein that is capable of binding cellulose with high

affinity and in a reversible manner. The CBD of the present invention may be used, for example, in the bio-immobilization of biologically active molecules to cellulose. The CBD of the present invention may be fused to a second protein to form a CBD fusion protein. The presence of a CBD protein in a CBD fusion protein allows for easy and selective purification of the CBD fusion protein by incubation with cellulose. Examples of second proteins include: Protein A, protein G, streptavidin, avidin, Taq polymerase and other polymerases, alkaline phosphatase, RNase, DNase, various restriction enzymes, peroxidases, glucanases such as endo-1,4-beta glucanase, endo-1,3-beta-glucanase, chitinases, and others, beta and alfa glucosidases, beta and alpha glucoronidases, amylase, transferases such as glucosyl-transferases, phospho-transferases, chloramphenicol-acetyl-transferase, beta-lactamase and other antibiotic modifying and degrading enzymes, luciferase, esterases, lipases, proteases, bacteriocines, antibiotics, enzyme inhibitors, different growth factors, hormones, receptors, membranal proteins, nuclear proteins, transcriptional and translational factors and nucleic acid modifying enzymes. Specifically, the CBD protein may be fused to an antibody or an antigenic determinant to form a CBD fusion product that is useful in diagnostic kits and in immunoassays.

Thus, for example, bodily fluids can be tested for the presence of particular antibodies (*e.g.*, heat shock protein (HSP) antibody) by making use of a CBD and an HSP epitope. Conversely, an HSP protein, a cross-reactive HSP-related protein, or antigenic portions thereof can be detected using a CBD-HSP antibody fusion protein.

The term "CBD" or "CBD protein" or "cellulose binding domain protein" refers to a protein comprising the amino acid sequence shown in Figure 1 and includes functional homologs and functional derivatives thereof, provided that the functional homolog or functional derivative possesses the capability of binding to cellulose with high affinity and in a reversible manner. The CBD of the present invention is provided substantially free of other proteins with which it is naturally associated, for instance, the balance of the major CbpA protein, discussed above. In addition, one or more predetermined amino acid residues in the polypeptide may be substituted, inserted, or deleted, for example, to produce a CBD having improved biological properties, or to vary binding and expression levels. Some of the desired CBD proteins falling within the scope of the present invention may optionally possess covalent or non-covalent modifications of the naturally occurring molecule, including, but not limited to, glycosylation modifications. Through the use of recombinant DNA technology, the CBD proteins of the present invention having residue deletions, substitutions and/or insertions may be prepared by altering the underlying nucleic acid. The modifications or mutations that may be made in the DNA encoding the CBD of the present invention must not alter the reading frame and preferably will not create complementary regions that could produce secondary mRNA structure (*see*, European Patent Publication No. EP 75,444)

The CBD protein of the present invention is one having at least 70% homology to the amino acid sequence shown in Figure 1, preferably, at least 80% homology, more preferably, at least 90% homology, and

most preferably, at least 95% homology. The term "X% homology" is not intended to be limited to sequences having a X% homology over the entire length of the protein. The 70% homology is also intended to include X% homology occurring in identified functional areas within the CBD protein of Figure 1. An example of a functional area would be a defined set of amino acids having the ability to bind cellulose with high affinity and in a reversible manner. Such protein homologs may also be referred to herein as "CBD functional homologs." In one embodiment of the present invention, such a functional area may have about 100 amino acids. In another embodiment of the present invention, such a functional area may have about 50 amino acids. The most desirable CBD protein of the present invention is one comprised of the amino acid sequence shown in Figure 1.

The term "CBD functional derivative" as used herein refers to any "fragment", "variant", "analogue" or "chemical derivative" of the CBD protein amino acid sequence shown in Figure 1 which retains the capability of binding to cellulose with high affinity and in a reversible manner and is preferably between about 2 and about 160 amino acids in length, more preferably between about 25 and about 125 amino acids in length and most preferably between about 50 and about 100 amino acids in length.

The term "fragment" is used to indicate a CBD protein which is derived from the CBD protein shown in Figure 1, and has a naturally occurring sequence. Such a fragment may be produced by proteolytic cleavage of the full-length protein. Alternatively, the fragment is obtained recombinantly by appropriately modifying the DNA sequence encoding the CBD protein to delete one or more amino acids at

one or more sites of the C-terminal, N-terminal, and within the naturally occurring sequence. Fragments of the CBD protein can be screened for the ability to bind cellulose with high affinity and in a reversible manner to determine the identity or utility of a functional derivative.

The term "variant" as used herein is defined as a molecule in which the amino acid sequence, glycosylation pattern, or other feature of a naturally occurring molecule has been modified covalently or noncovalently and is intended to include mutants. Some of the variants falling within this invention possess amino acid substitutions deletions, and/or insertions provided that the final construct possesses the desired ability of binding cellulose with high affinity and in a reversible manner. Amino acid substitutions in the CBD protein may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups or nonpolar head groups having similar hydrophilicity values include the following: leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine. Also included within the definition of variant are those proteins having additional amino acids at one or more sites of the C-terminal, N-terminal, and within the naturally occurring CBD sequence as long as the variant retains the capability of binding cellulose with high affinity and in a reversible manner.

The term "chemical derivative" as used herein refers to a CBD protein produced by chemical modification of naturally occurring or variant CBD protein. Illustrative of an example of a chemical modification would be replacement of H by an alkyl, acyl, or amino group.

The phrase "binding cellulose with high affinity" as used herein refers to the ability of the CBD protein to bind to cellulose with a K_d in μM ranging from about 1.5 to about 0.5, preferably from about 1.4 to about 0.8. More preferably, the high affinity binding refers to the ability of the instant CBD to bind to crystalline cellulose with a K_d of about 1.1 or less, most preferably about 1.0 or less.

The phrase "binding cellulose in a reversible manner" as used herein refers to the ability of the CBD protein to be released from the cellulose-CBD protein binding complex by releasing agents or solutions, such as 6M urea, 6M guanidine-HCl and other denaturing reagents, including nonionic surfactants. Preferably, however, those denaturing reagents are used which allow the released CBD or fusion product to be reconstituted. For example, the CBD may be reconstituted from the treatment with 6M urea or 6M quanidine-Hcl by subjecting the denatured protein to renaturing conditions described in Sections 6.1.4, 7.1.1, 7.2.1, and 8.1.4, below.

The term "CBD fusion protein" as used herein refers to the joining together of at least two proteins, a CBD protein and a second protein. In some embodiments of the present invention, the second protein may be fused or joined to a third protein. In the present invention, examples of second proteins include enzymes, such as nucleic acid modification enzymes, proteases, hormones or hormone precursors,

polypeptides, peptides, antibodies, antigens, antigenic epitopes and variants thereof. In some preferred embodiments of the present invention, the second protein is Protein A; in other preferred embodiments of the present invention, the second protein is an HSP protein. One preferred embodiment of the present invention is a fusion protein comprised of CBD protein, Protein A or anti-HSP recombinant IgG. The CBD fusion protein of the present invention may comprise an enzymatic or chemical cleavage site upstream and preferably adjacent to the N-terminus of the second protein and/or an enzymatic or chemical cleavage site downstream and preferably adjacent the C-terminus of the second protein thereby providing a means for recovering the second protein from the CBD fusion protein through use of a cleaving agent.

The term "CBD fusion protein-cellulose binding complex" as used herein refers to the complex formed when cellulose binds the CBD protein of a CBD fusion protein.

"Nucleic acid" refers to a nucleotide sequence comprising a series of nucleic acids in a 5' to 3' phosphodiester linkage that may be either an RNA or a DNA sequence. If the nucleic acid is DNA, the nucleotide sequence is either single or double stranded. CBD protein encoding nucleic acid is RNA or DNA that encodes a CBD protein capable of binding cellulose with high affinity, is complementary to nucleic acid sequence encoding such CBD protein, or hybridizes to nucleic acid sequence encoding such CBD protein and remains stably bound to it under stringent conditions.

The phrase "nucleic acid encoding the CBD protein of the present invention" includes nucleic acid of genomic, cDNA, synthetic, and semi-synthetic

origin which, by virtue of its origin or manipulation, is not associated with any portion of the polynucleotide to which it is associated in nature, and may be linked to a polynucleotide other than that to which it is linked in nature, and includes single or double stranded polymers of ribonucleotides, deoxyribonucleotides, nucleotide analogs, or combinations thereof, as long as the CBD being encoded retains its ability to bind cellulose with high affinity. The phrase also includes various modifications known in the art, including but not limited to radioactive and chemical labels, methylation, caps, internucleotide modifications such those with charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, etc.) and uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoramidites, carbamites, etc.), as well as those containing pendant moieties, intercalators, chelators, etc. as long as the CBD encoded by the nucleic acid retains the ability to bind cellulose with high affinity and in a reversible manner.

CBD encoding nucleic acid may be used to construct recombinant expression vectors capable of expressing the CBD protein or the CBD fusion protein of the present invention. A nucleic acid construct is capable of expressing a protein if it contains nucleotide sequences containing transcriptional and translational regulatory information and such sequences are "operably linked" to nucleotide coding sequences. "Operably linked" refers to a linkage in which the regulatory DNA sequences and the DNA sequence to be expressed are connected in such a way as to permit transcription and ultimately translation.

In constructing the CBD fusion protein expression vector, the nucleic acid encoding the CBD protein will

be linked or joined to the nucleic acid encoding the second protein such that the open reading frame of the CBD protein and the second protein is intact, allowing translation of the CBD fusion protein to occur. CBD nucleic acid may be obtained from a variety of cell sources that produce cellulose binding domains that bind with high affinity and in a reversible manner or that produce CBD encoding mRNA. The preferred source of CBD encoding nucleic acid is *Clostridium cellulovorans*. The CBD encoding nucleic acid may be obtained as described in Section 6.1.

The nucleic acid encoding the CBD protein of the present invention may be obtained from isolated and purified DNA from cell sources or by genomic cloning. Either cDNA or genomic libraries of clones may be prepared using techniques well known in the art and may be screened for particular CBD encoding nucleic acid with nucleotide probes that are substantially complementary to any portion of the gene. If detection of CBD protein encoding conserved nucleotide regions is desired, nucleotide probes should be based on CBD nucleotide sequences conserved from species to species. If detection of CBD protein encoding unique nucleotide regions is desired, nucleotide probes should be based on unique CBD nucleotide sequences. Alternatively, cDNA or genomic DNA may be used as templates for PCR cloning with suitable oligonucleotide primers. Full length clones, *i.e.*, those containing the entire coding region of the desired CBD protein may be selected for constructing expression vectors, or overlapping cDNAs can be ligated together to form a complete coding sequence. Alternatively, CBD-encoding DNAs may be synthesized in whole or in part by chemical synthesis using techniques deemed to be standard in the art.

Many vectors are available, and selection of the appropriate vector will depend on 1) whether it is to be used for nucleic acid amplification or for nucleic acid expression, 2) the size of the nucleic acid to be inserted into the vector, and 3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of nucleic acid or expression of nucleic acid) and the host cell for which it is compatible.

The term "host cell" refers to those cells capable of growth in culture and capable of expressing a CBD protein or CBD fusion protein. The host cells of the present invention encompass cells in *in vitro* culture and include procaryotic, eucaryotic, and insect cells. A host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers (*e.g.*, zinc and cadmium ions for metallothionine promoters. Therefore expression of the CBD protein or CBD fusion protein may be controlled. The ability to control expression will be important if the CBD protein or CBD fusion protein is lethal to a host cell. Modifications (*e.g.*, phosphorylation) and processing (*e.g.*, cleavage) of protein products are important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of protein. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the CBD protein or CBD fusion protein expressed. Preferably, the host

cell should secrete minimal amounts of proteolytic enzymes.

In the present invention, a host cell is provided comprised of nucleic acid encoding the CBD protein or CBD fusion protein of the present invention that is capable of binding to cellulose with high affinity. The preferred host cell for cloning and expression of the CBD proteins of the present invention is a prokaryotic cell. Procaryotes are particularly useful for rapid production of large amounts of nucleic acid, for production of single-stranded nucleic acid templates used for site-directed mutagenesis, for screening many mutants simultaneously, and for nucleic acid sequencing of the mutants generated. An example of a prokaryotic cell useful for cloning and expression of the CBD protein of the present invention is *E. coli* strain XL1-blue from Stratagene. Another example of a prokaryotic cell useful for cloning and expression of the CBD fusion protein is *Staphylococcus aureus*. Yet another example of a system useful for cloning and expression of the CBD protein or CBD fusion proteins is a *Pichia* expresion kit from Invitrogen Corporation (San Diego, CA).

Various expression vector/host systems may be utilized equally well by those skilled in the art for the recombinant expression of CBD proteins and CBD fusion proteins. Such systems include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the desired CBD coding sequence; yeast transformed with recombinant yeast expression vectors containing the desired CBD coding sequence; insect cell systems infected with recombinant virus expression vectors

(*e.g.*, baculovirus) containing the desired CBD coding sequence; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing the desired CBD coding sequence; or animal cell systems infected with recombinant virus expression vectors (*e.g.*, adenovirus, vaccinia virus) including cell lines engineered to contain multiple copies of the CBD nucleic acid either stably amplified (*e.g.*, CHO/dhfr, CHO/glutamine synthetase) or unstably amplified in double-minute chromosomes (*e.g.*, murine cell lines).

Vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. The expression elements of these vectors vary in their strength and specificities. Depending on the host/vector system utilized, any one of a number of suitable transcription and translation elements may be used. For example, when cloning in procaryotic cell systems, promoters isolated from the genome of procaryotic cells, (*e.g.*, the bacterial tryptophane promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the inserted sequences.

A signal sequence may be a component of the vector, or it may be a part of the CBD nucleic acid that is inserted into the vector. The signal sequence may be the naturally occurring one preceding the CBD sequence or a non-naturally occurring sequence. The signal sequence should be one that is recognized and processed by the host cell. An origin of replication

refers to the unique site of initiation of replication of a host organism. It is desirable for cloning and expression vectors to comprise a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics or other toxins, *e.g.* ampicillin; complement auxotrophic deficiencies; or supply critical nutrients not available from complex media. One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen.

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the nucleic acid encoding the polypeptide of interest. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as that encoding a CBD protein or CBD fusion protein, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from nucleic acid under their control in response to some change in culture conditions, *e.g.*, the presence or absence of a nutrient or a change in temperature. At this time, a large number of promoters recognized by a

variety of potential host cells are well known. These promoters are operably linked to nucleic acid encoding the polypeptide of interest by removing the promoter from the source nucleic acid by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the naturally occurring promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the polypeptide of interest. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed polypeptide of interest as compared to the naturally occurring promoter. In general, plasmid vectors containing promoters and control sequences which are derived from species compatible with the host cell are used with these hosts. The vector ordinarily carries a replication site as well as marker sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (Bolivar, *et al.*, Gene 2:95 [1977]). The pBR322 plasmid contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid, must also contain or be modified to contain promoters and other control elements commonly used in recombinant DNA construction.

Promoters suitable for use with prokaryotic hosts illustratively include the β-lactamase and lactose promoter systems (Chang *et al.*, Nature, 275:615 [1978]; and Goeddel *et al.*, Nature 281:544 [1979]), alkaline phosphatase, the tryptophan (trp) promoter system (Goeddel Nucleic Acids Res. 8:4057 [1980] and EPO Appln. Publ. No. 36,776) and hybrid

promoters such as the tac promoter (H. de Boer *et al.*, Proc. Natl. Acad. Sci. USA 80:21-25 [1983]). However, other functional bacterial promoters are suitable. Their nucleotide sequences are generally known, thereby enabling a skilled worker operably to ligate them to nucleic acid encoding prorelaxin (Siebenlist *et al.*, Cell 20:269 [1980]) using linkers or adapters to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the nucleic acid encoding prorelaxin.

Expression vectors used in prokaryotic host cells will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA.

Construction of suitable vectors containing one or more of the above listed components and including the desired coding and control sequences employs standard ligation techniques. Isolated plasmids or nucleic acid fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

Particularly useful in the practice of this invention are expression vectors that provide for the expression of prokaryotic cells of nucleic acid encoding the CBD protein. In general, expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate

for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

"Transformation" means introducing nucleic acid into an organism so that the nucleic acid is replicable, either as an extrachromosomal element or by chromosomal integration. Unless indicated otherwise, the method used herein for transformation of the host cells is the method of calcium treatment using calcium chloride as described by Cohen, F.N. *et al.*, Proc. Natl. Acad. Sci. (USA),69:2110 (1972).

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction and/or sequenced by the method of Messing *et al.*, Nucleic Acids Res. 9:309 (1981) or by the method of Maxam *et al.*, Methods in Enzymology 65:499 (1980).

Host cells may be transformed with the expression vectors of this invention and cultured in conventional nutrient media modified as is appropriate for inducing promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

Prokaryotic cells used to produce the polypeptide of this invention are cultured in suitable media as described generally in Sambrook, *et al.* (1989) Electrophoresis buffers *in* Molecular Cloning (Nolan, C. ed.), Cold Spring Harbor Laboratory Press, NY, pp. B.23-24; Sambrook *et al.* (1989) Bacterial

Media *in* Molecular Cloning (Nolan, C. ed.), Cold Spring Harbor Laboratory Press, NY, pp. A.1-4.

The selection of host cells producing a CBD protein or CBD fusion protein of the present invention may be identified by at least four general approaches:

(a) DNA-DNA, DNA-RNA or RNA antisense RNA hybridization: the presence of nucleic acid coding for CBD proteins of the present invention can be detected by nucleic acid hybridization using hybridization probes and/or primers for PCR reactions comprising nucleotides that are homologous to the CBD coding sequence;

(b) the presence or absence of "marker" gene functions: the selection of host cells having nucleic acid encoding CBD protein of the present invention can be identified and selected based upon the presence or absence or certain marker gene functions, e.g., resistance to antibiotics. For example, if the CBD coding sequence is inserted within a marker gene sequence of the cloning or expression vector, recombinants containing that coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the CBD nucleic acid sequence under the control of the same or different promoter used to control the expression of the CBD coding sequence. Expression of the marker in response to induction or selection indicates expression of the CBD coding of the CBD coding sequence.

(c) assessing the level of transcription as measured by the expression of CBD protein or CBD fusion protein mRNA transcripts in the host cell: transcriptional activity of the CBD coding region can be assessed by hybridization assays. For example, polyadenylated RNA can be isolated and analyzed by

Northern blot using a probe homologous to the CBD coding sequence or particular portions thereof. Alternatively, total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes; and

(d) detection of the CBD protein or CBD fusion protein as measured by immunoassay and, by the ability of the protein to bind cellulose with high affinity and in a reversible manner. The expression of CBD proteins can be assessed immunologically, for example by Western blots or by immunoassays such as RIAs. The expression of CBD protein can be assayed by the ability of the expressed protein to bind cellulose with high affinity and in a reversible manner.

The expressions "cell" and "cell culture" are used interchangeably and all such designations include progeny and ancestors. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the cell are included.

The phrase "stringent conditions" as used herein refers to those hybridizing conditions that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS at 50 °C.; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42 °C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M Sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS, and 10% dextran

sulfate at 42 °C, with washes at 42 °C in 0.2 x SSC and 0.1% SDS.

The term "recovery" as used herein refers to the ability of the cellulose CBD protein complex to release the CBD protein from the cellulose-CBD protein binding complex under certain conditions, which conditions include the use of releasing agents, for example, denaturing reagents, such as 6M urea or 6M guanidine-HCl. Any releasing agent that has the ability of releasing the CBD protein from the cellulose-CBD protein binding complex can be used to recover the CBD protein. Preferably, the CBD is only temporarily denatured and not irreversibly degraded by treatment with the releasing agent. Thus, the CBD is recovered by reconstituting the eluted protein, as described in Section 7.1.1, 7.2.1, or 8.1.4.

The use of the phrase "cleaving agents" as used herein refers to a reagent used to cleave the CBD protein or CBD fusion protein specifically so as to release or excise certain components, such as the second protein of a CBD fusion protein, as desired. Suitable cleaving agents herein include enzymes, such as endoproteases, prohormone convertases, *e.g.*, PC1, PC2, furin, Kex2, subtilisin, or its mutants; and chemical agents, such as organic and inorganic acids, hydroxylamine, N-bromosuccinimide, and cyanogen bromide. Hydrolysis of peptide bonds catalyzed by a variety of proteolytic enzymes is taught in The Enzymes, 3rd Edition, Boyer, Ed., Academic Press, Vol. III [1971]; Meth. Enzymology., Vol. XIX, Perlman and Lorand, Ed., New York: Academic Press [1970]; Meth. Enzymol., Vol. XLV, Lorand, Ed. New York: Academic Press [1976]; Drapeau, J. Biol.Chem., 253:5899-5901 [1978] and Drapeau, Meth. Enzymology., 47:89-91[1977]. For an extensive listing of chemical agents, see

Witcop in Advances in Protein Chemistry, Anfinsen *et al.*, ed., 16:221-321, Academic Press, New York [1961], including Table III on p. 226. Other cleavage agents suitable herein are deemed to be understood by those skilled in the art keeping in mind the desired junction for cleavage and whether the reagent can act on reduced or oxidized forms of CBD fusion proteins. Conditions used for cleavage of the CBD fusion protein will depend on the cleavage agent employed, and the conditions will be readily apparent to one skilled in the art given the cleavage agent employed.

The CBD fusion protein of the present invention is designed and constructed to comprise the codon(s) necessary to achieve cleavage by the desired cleaving agent at desired positions, *i.e.* upstream, preferably adjacent the N-terminus of the second protein of the CBD fusion protein or downstream, and preferably adjacent the C-terminus of the second protein or both if the second protein of the fusion protein is an internal amino acid of the fusion protein.

The term "glycosylation" and grammatical derivatives as used herein refers to the post-translational modification process of adding a series of sugar residues to proteins to produce glycoproteins. Glycosylation can occur in the cytosol, the endoplasmic reticulum, or the Glogi apparatus of mammalian cells. Alternatively, glycosylation can be accomplished by synthetic methods, for example by providing an appropriate glycosyl donor. *See, e.g.*, Kahne, *et al.* J. Am. Chem. Soc., 111:6881-2 [1989].

This invention also relates to diagnostic detection of proteins of interest in test samples, especially in biological samples, such as tissue

extracts or biological fluids, such as serum or urine through use of the CBD fusion protein of the present invention. The biological samples are preferably of mammalian origin and most preferably of human origin. A preferred protein of interest to be detected in a mammalian biological sample is an HSP protein, an HSP antibody, cross-reactive HSP-related proteins, or antigenic portions thereof. The presence of the HSP antibody in a mammalian biological sample, for example, may be predictive or indicative of insulin-dependent diabetes mellitus (IDDM). In one embodiment of the present invention, the CBD Protein A fusion protein is comprised of a third protein, an IgG antibody, for example, IgG anti-HSP, which is used to detect the presence of an antigen, for example HSP, in biological samples using a variety of immunoassay formats well known in the art. Alternatively, the second protein of the CBD fusion protein is comprised of an antigenic determinant, an epitope, useful in the detection of antibodies that recognize the antigenic determinant. A preferred epitope is the HSP protein.

Protein A is a protein found in the cell wall of *Staphylococcus aureus* that binds to the Fc portion of IgG molecules and thus precipitates the antibodies. Protein A has utility in immunoassays, such as an RIA or ELISA, where it is used to isolate antibodies or antigen-antibody complexes.

In the present invention, Protein A is a preferred second protein of a CBD fusion protein. A CBD-Protein A fusion protein has utility in diagnostic immunoassays that detect the presence of or measure the quantity or concentration of an antibody or an antibody-antigen complex.

A CBD-Protein A fusion protein of the present invention also has utility in a diagnostic kit

comprised of cellulose and a CBD-fusion protein wherein the CBD fusion protein component retains its ability to bind both cellulose and IgG of a second component, for example, an antibody-antigen complex or an antibody. The CBD fusion protein of the present invention also has utility as a means for affinity purification of antibodies or antigenic determinants, *i.e.* epitopes. A preferred antigenic determinant of the present invention is the HSP protein, related protein or antigenic portion thereof. Preferred second proteins of a CBD fusion protein include HSP protein or anti-HSP IgG. In the present invention, CBD-HSP epitope fusion proteins find utility in immunoassays designed to measure quantities of HSP antibody found in the serum of human mammals.

In another embodiment of the present invention the CBD-fusion protein comprises a CBD component which retains its ability to bind cellulose or chitin and a second component which is an enzyme which retains its catalytic activity. The CBD-fusion proteins of this embodiment have utility as a means to permit enzyme catalyzed reactions on a solid substrate of cellulose or chitin. As described above, the CBD-enzyme fusion proteins can be prepared either by conjugating purified CBD to a purified enzyme preparation by standard chemical methods or by expression of CBD and enzyme encoding nucleic acids by recombinant methods. In one example of this embodiment of the invention, the second component of the CBD-fusion protein is a heparinase enzyme. The CBD-heparinase can be immobilized on a cellulose substrate and catalyzes conversion of heparin to component saccharide moieties.

Yet another aspect of the present invention relates to the ability of CBD to modify the growth of

plant tissues. Such ability finds application in the agricultural industry, for example, where it may be desirable to promote pollen tube growth and/or root growth. In addition, since CBD in high concentration has the ability to inhibit root growth it is useful to prevent the devlopment of undesirable plants, for example, "weed" plants. In another example, the CBD finds application in enhancing growth of fungi, such as mushrooms, wherein the CBD binds chitin and stimulates the growth and/or development of developing basidiocarps or fruiting bodies.

Yet another aspect of the present invention relates to the ability of CBD to bind to chitin of insect exoskeletons and other insect parts, including the mid-gut. Such ability finds application in the use of CBD as a bio-pesticide wherein the CBD is linked to a microorganism useful for control insect pests, such as *Bacillus thuringiensis* (BT) or other microorganism expressing the BT toxin gene, or such as a chitinase secreting fungus. The CBD functions to bind the microorganism to the chitinous portions of the insect where the microbially produced toxin or enzyme leads to death of the insect pest. In one mode of this embodiment of the invention, the CBD-microorganism complex can be applied directly to plant parts suspected or at risk of infestation by insect pests or, alternatively, the CBD-microorganism complex can be immobilized on bait stations which are set out in the infested fields or on plants infested with insect pests. In another alternative mode of this embodiment of the invention a CBD-microorganism complex is applied to wood or cellulose-based products to prevent or control infestation by wood destroying insect pests. For example, a CBD-microorganism complex is applied to telephone poles or wood or

cellulose-based construction components where the CBD component binds tightly to invading insects and the microorganism of the complex is effective to control the insect pests.

Still another aspect of the present invention relates to the ability of CBD to bind to chitin found in fungi, including fungi which are useful for bioremediation or degradation of toxic environmental pollutants. According to one mode of this embodiment, a CBD fusion protein is used in which the second component is an enzyme which degrades the environmental pollutant. The CBD-enzyme fusion protein can be immobilized or a cellulose substrate and the device used to remove the pollutant from the environment by enzymatic degradation, for example, by placing the device in a stream of surface or underground water. According to any alternative mode of this embodiment of the invention, the CBD is bound to a fungus which secretes an enzyme which is useful for biomediation *i.e.*, which degrades an environmental pollutant such as a pesticide. The CBD-fungus complex is then immobilized on a cellulose substrate and provides a useful device to enzymatically degrade an environmental pollutant such as a pesticide including DDT or such as wood containing products left over from logging or saw milling operations. In one illustrative example, CBD is complexed with a white rot fungus, for example *Phanerochaete chrysosporium*, which produces lignin peroxidase, manganese peroxidase and degrades the pesticide DDT. The CBD-fungus complex is useful for bioremediation, for example, to remove a pesticide such as DDT from the environment.

The "antibody" as used herein is meant to include polyclonal antibodies, monoclonal antibodies (MAbs), humanized or chimeric antibodies, single chain

antibodies, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. An epitope refers to an antigenic determinant of an antigenic molecule.

The term IgG refers to a class of antibodies. IgG is a tetramer containing two light chains and two heavy chains that represents 80% of all immunoglobulins.

The term "detectable label" as used herein refers to any label which provides directly or indirectly a detectable signal and includes, for example, enzymes, radiolabelled molecules, fluoresors, particles, chemiluminesors, enzyme substrates or co-factors, enzyme inhibitors, magnetic particles. Examples of enzymes useful as detectable labels in the present invention include alkaline phosphatase and horse radish peroxidase. A variety of methods are available for linking the detectable labels to proteins of interest and include for example the use of a bifunctional agent, such as 4,4'-difluoro-3,3'-dinitro-phenylsulfone, for attaching an enzyme, for example, horse radish peroxidase, to a protein of interest. The attached enzyme is then allowed to react with a substrate yielding a reaction product which is detectable.

Falling within the scope of the present invention is a signal amplification method wherein the use of a detectable label comprised of a CBD protein allows for detection of femtogram quantities of the substance of interest. In this method, a first CBD protein is part of a CBD fusion product that is incubated with a cellulose fiber under conditions suitable for formation of a cellulose-CBD fusion product binding complex. Excess labeled CBD, for example, CBD fused or bound to an enzyme, such as

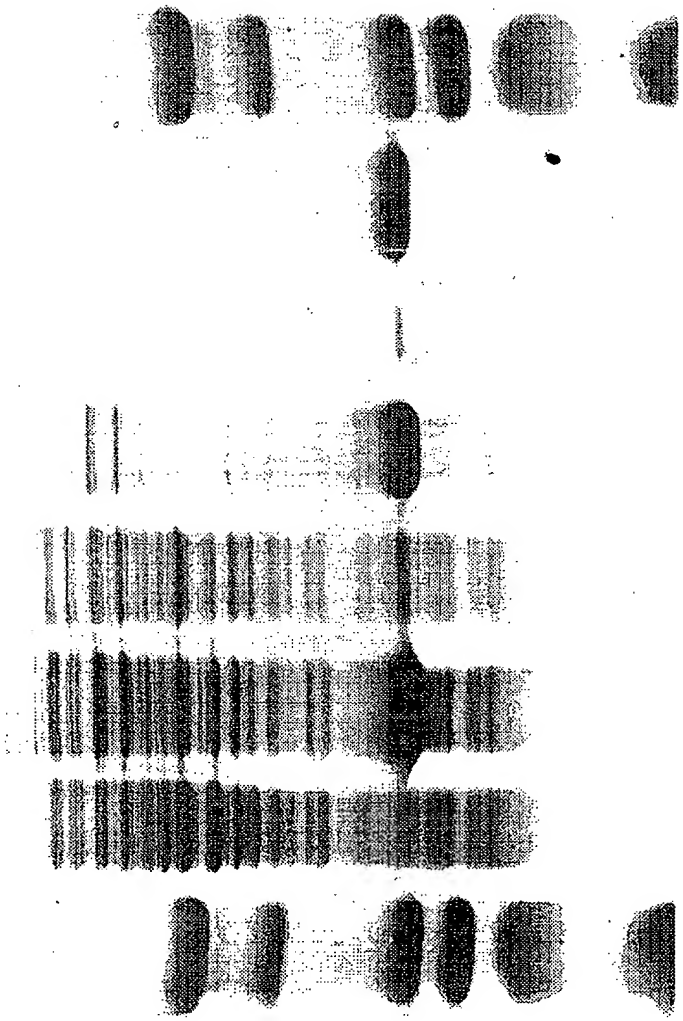

FIG.3

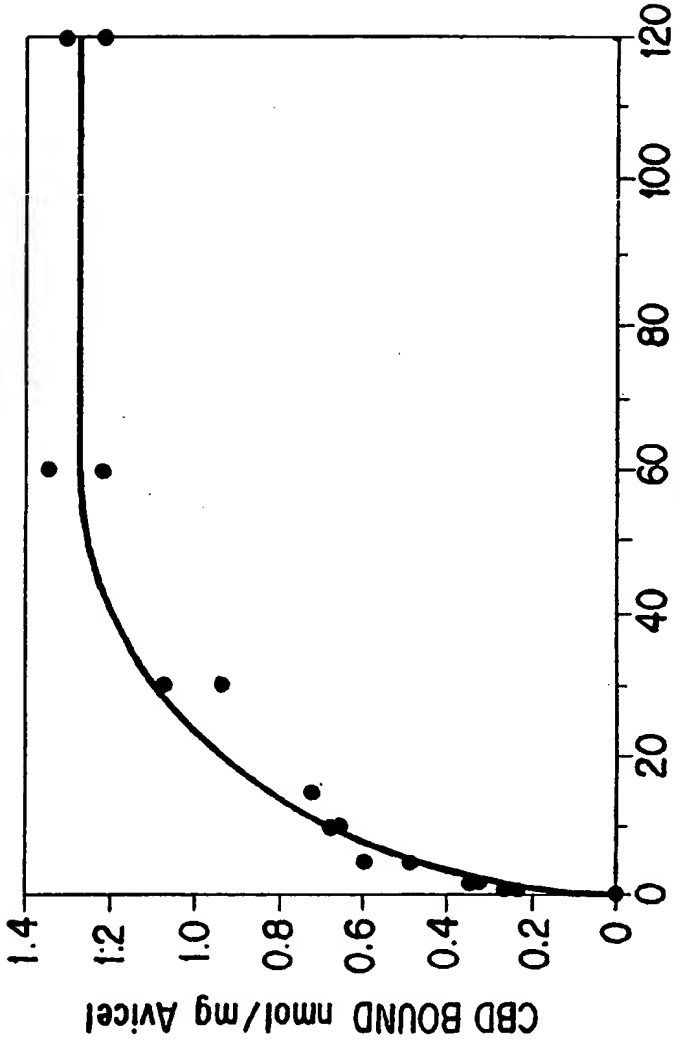
CBD BOUND nmol/mg Avicel
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
TIME (min)

FIG.4

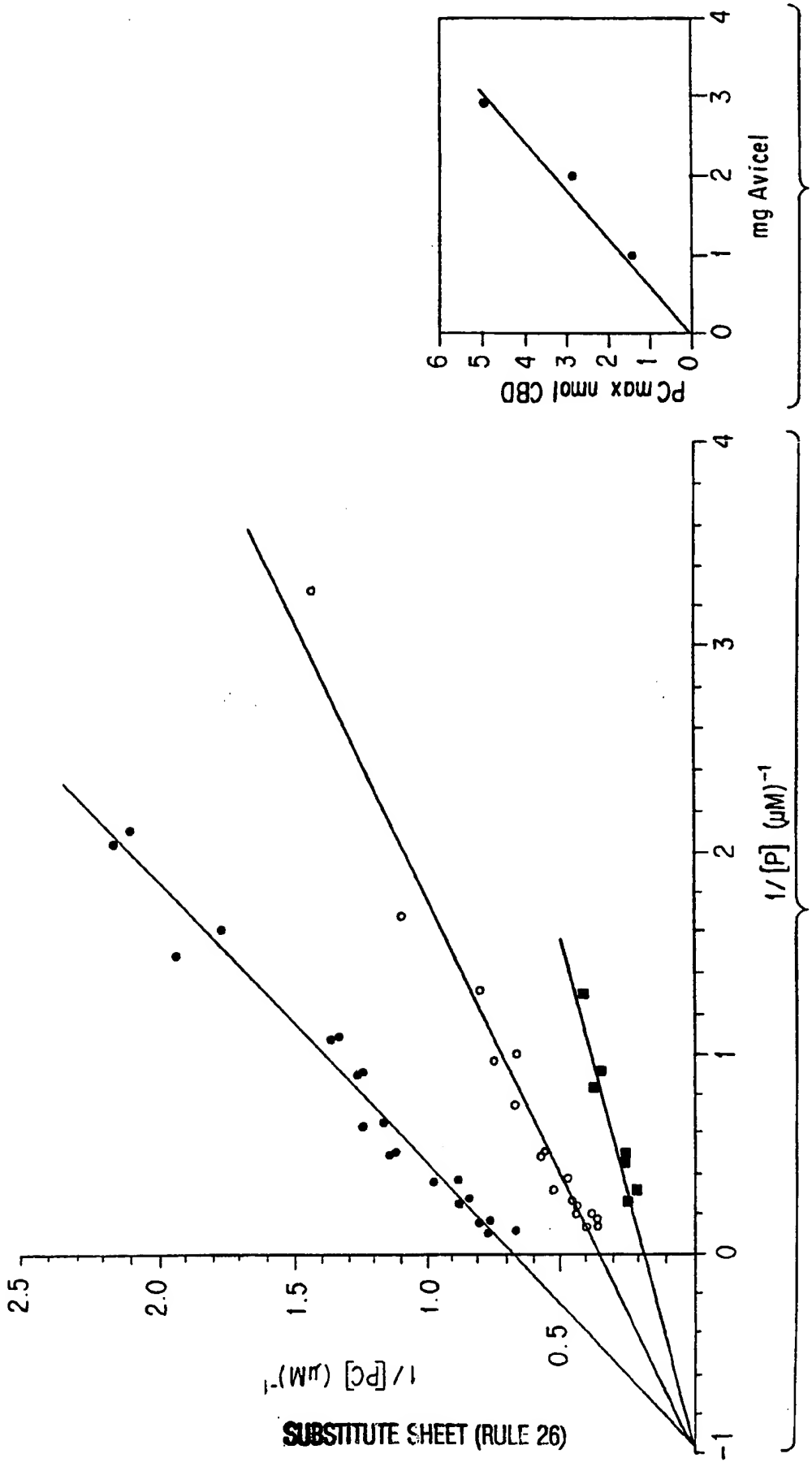

FIG. 5B

FIG. 5A

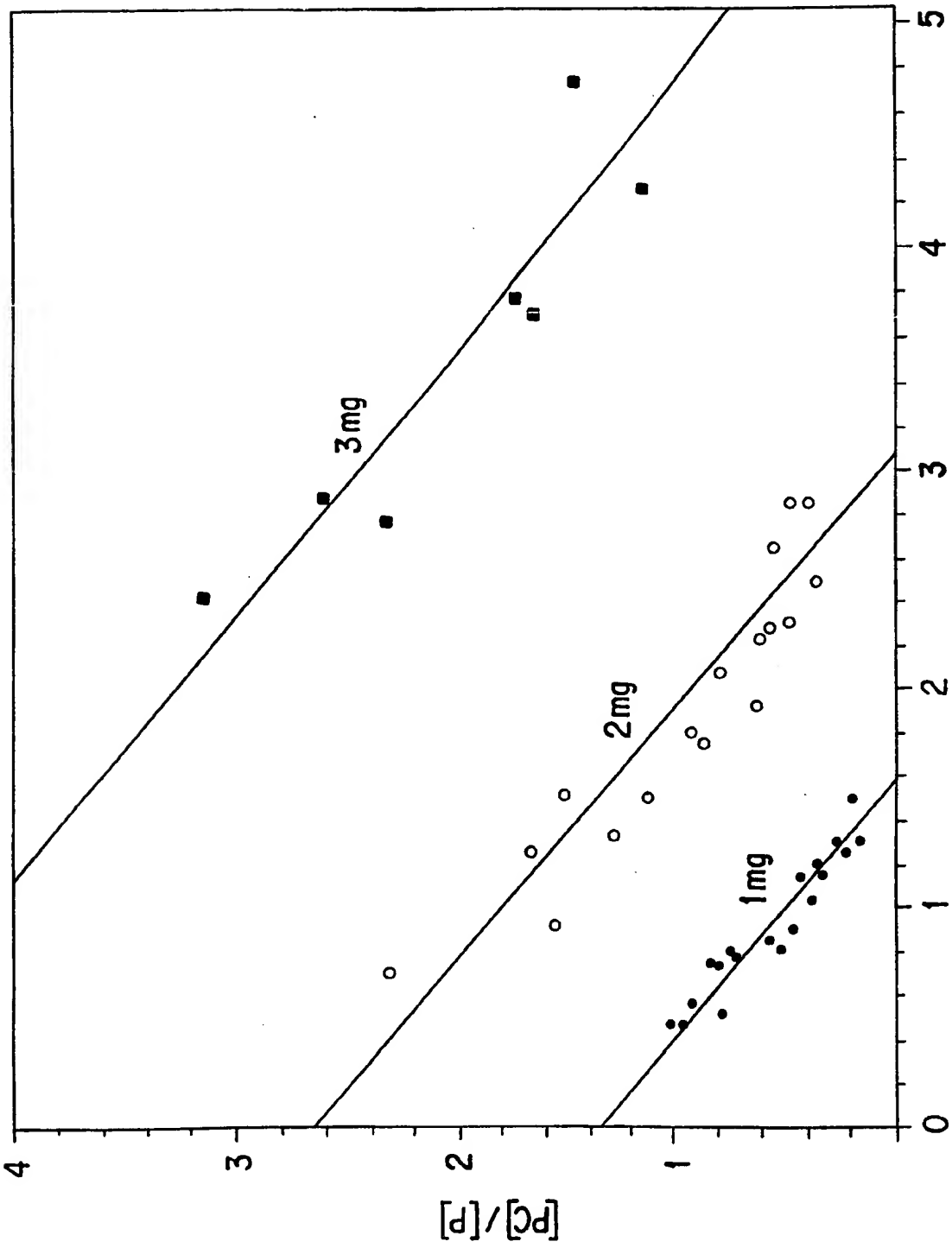

FIG.6

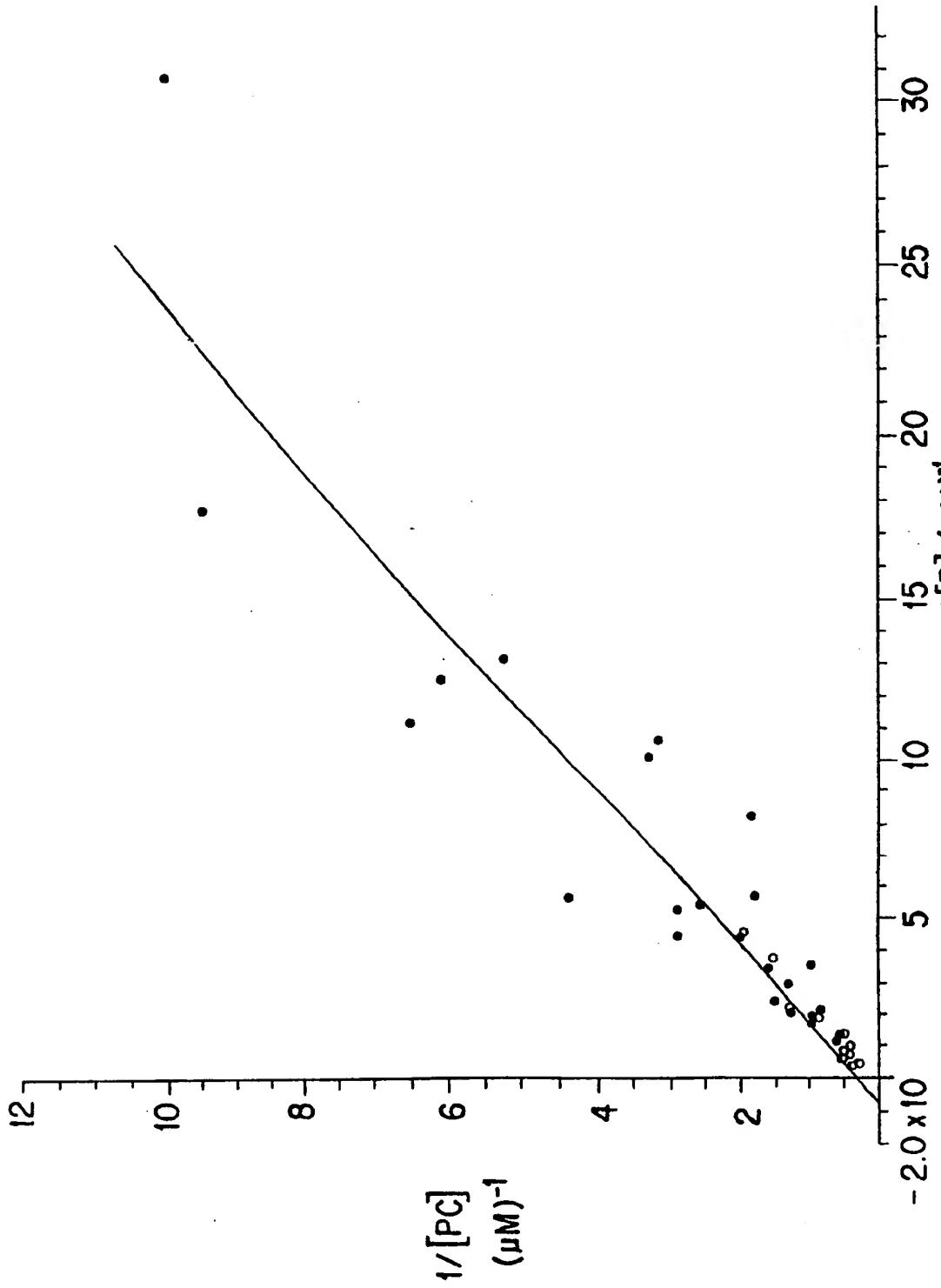

FIG.7

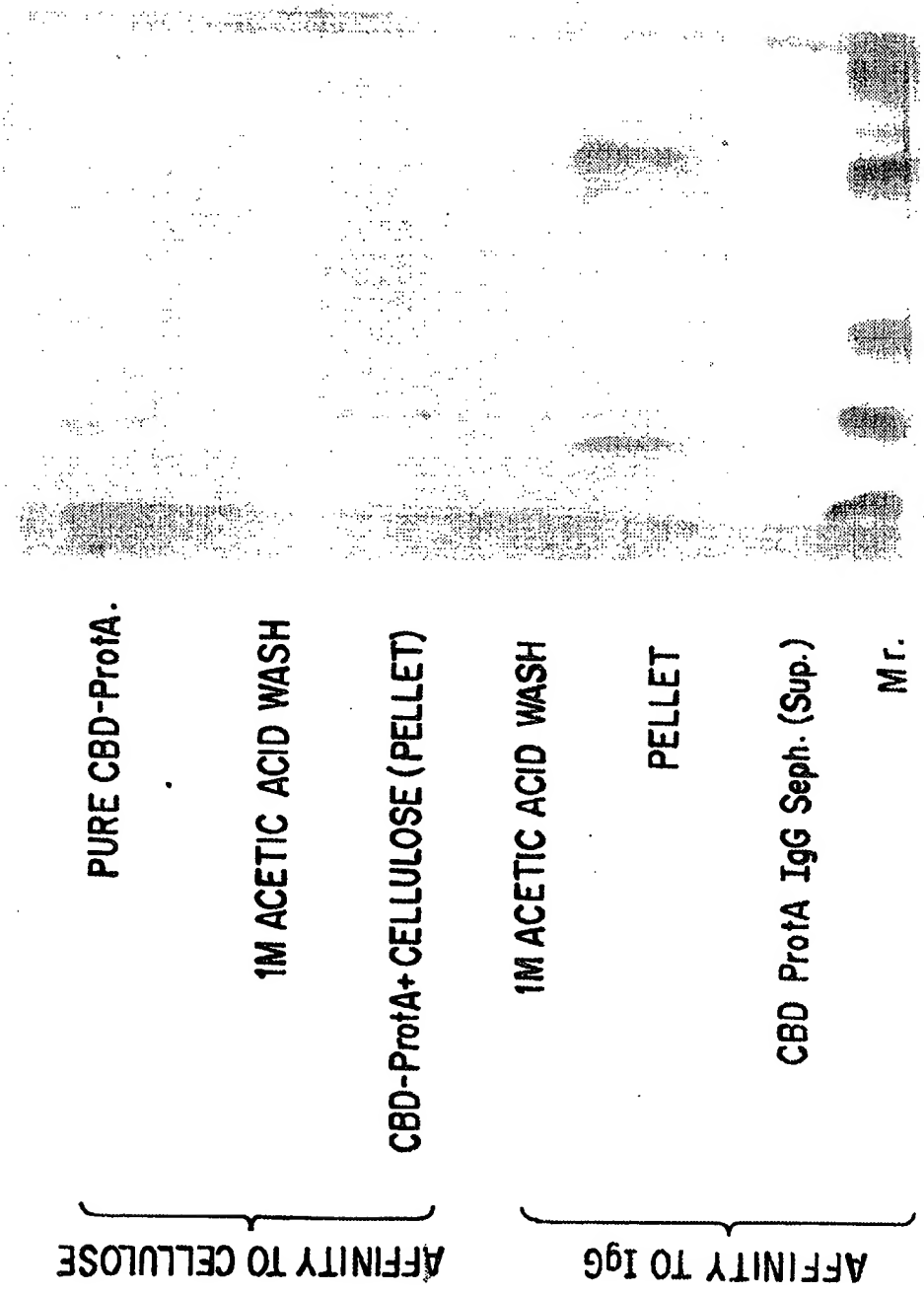
PURE CBD-ProtA.
1M ACETIC ACID WASH
CBD-ProtA+ CELLULOSE (PELLET)
1M ACETIC ACID WASH
PELLET
CBD ProtA IgG Seph. (Sup.)
Mr.
AFFINITY TO CELLULOSE
AFFINITY TO IgG

FIG.8

SUBSTRATE:	OBSERVED K_d (in μM)	OBSERVED PC_{max} (in μmoles CBD/g)
AVICEL PH101	1.1 $\pm$ 0.3	1.4 $\pm$ 0.3
SIGMACELL 20	1.1	1.2
SIGMACELL 50	1.4	1.7
SIGMACELL 100	1.3	0.5
MICROGRANULAR CELLULOSE	1.0	0.4
FIBROUS CELLULOSE	1.4	0.2
COTTON	0.8	6.4
CELLULON	1.2	5.3
XYLAN	–	0
SEPHADEX G-75	–	0
NIGERAN	–	0
CHITIN	1.0	1.6
AVICEL PH101		
+CMC (4mg/ml)	1.1	1.1
+CELLOBIOSE (4mg/ml)	1.0	1.5

FIG.9

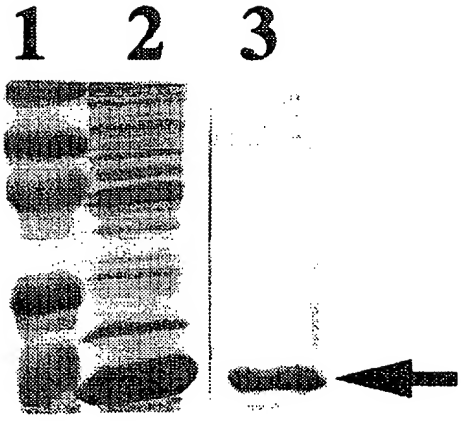

FIG. 10

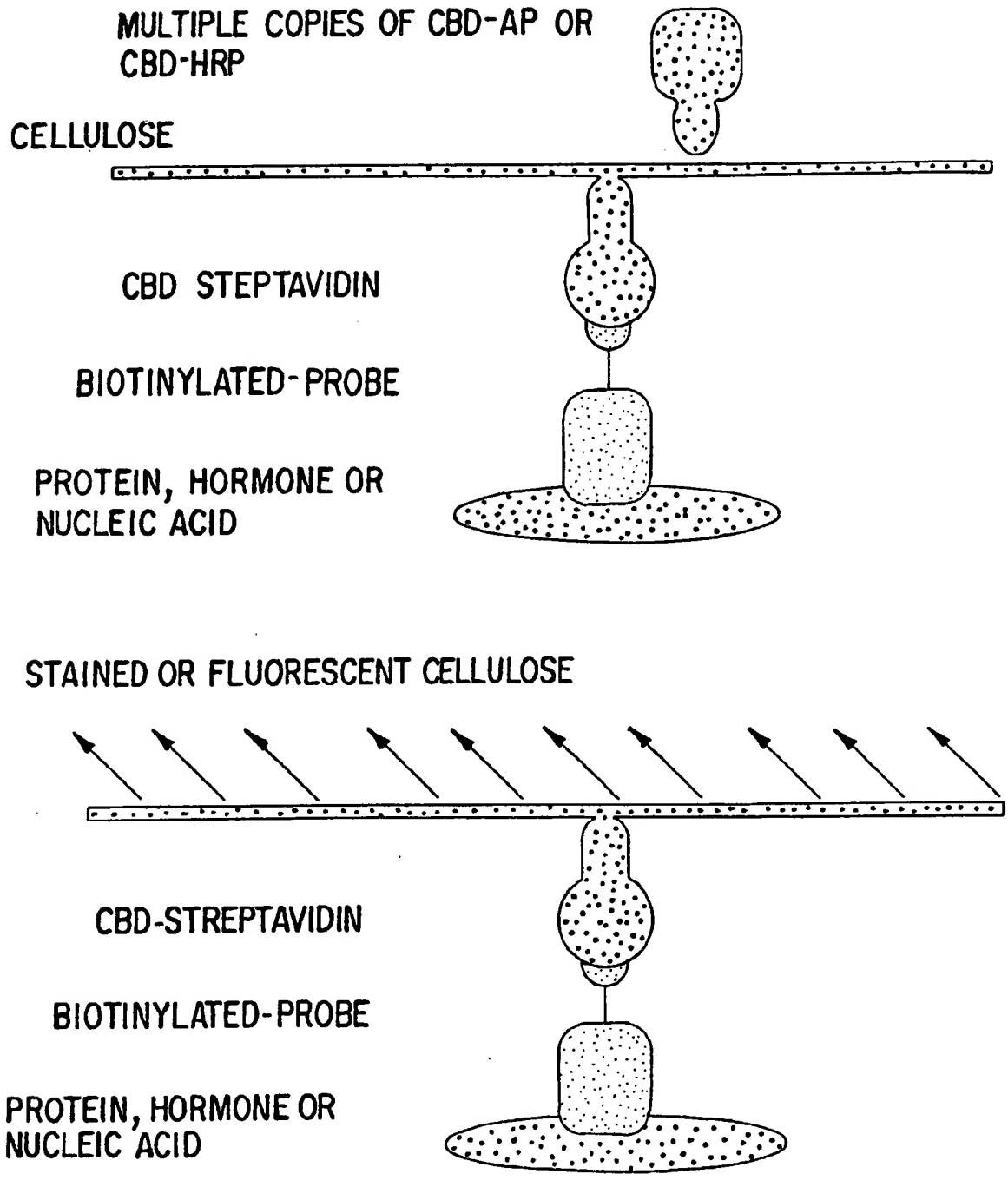
MULTIPLE COPIES OF CBD-AP OR CBD-HRP
CELLULOSE
CBD STEPTAVIDIN
BIOTINYLATED-PROBE
PROTEIN, HORMONE OR NUCLEIC ACID
STAINED OR FLUORESCENT CELLULOSE
CBD-STREPTAVIDIN
BIOTINYLATED-PROBE
PROTEIN, HORMONE OR NUCLEIC ACID

FIG. 11

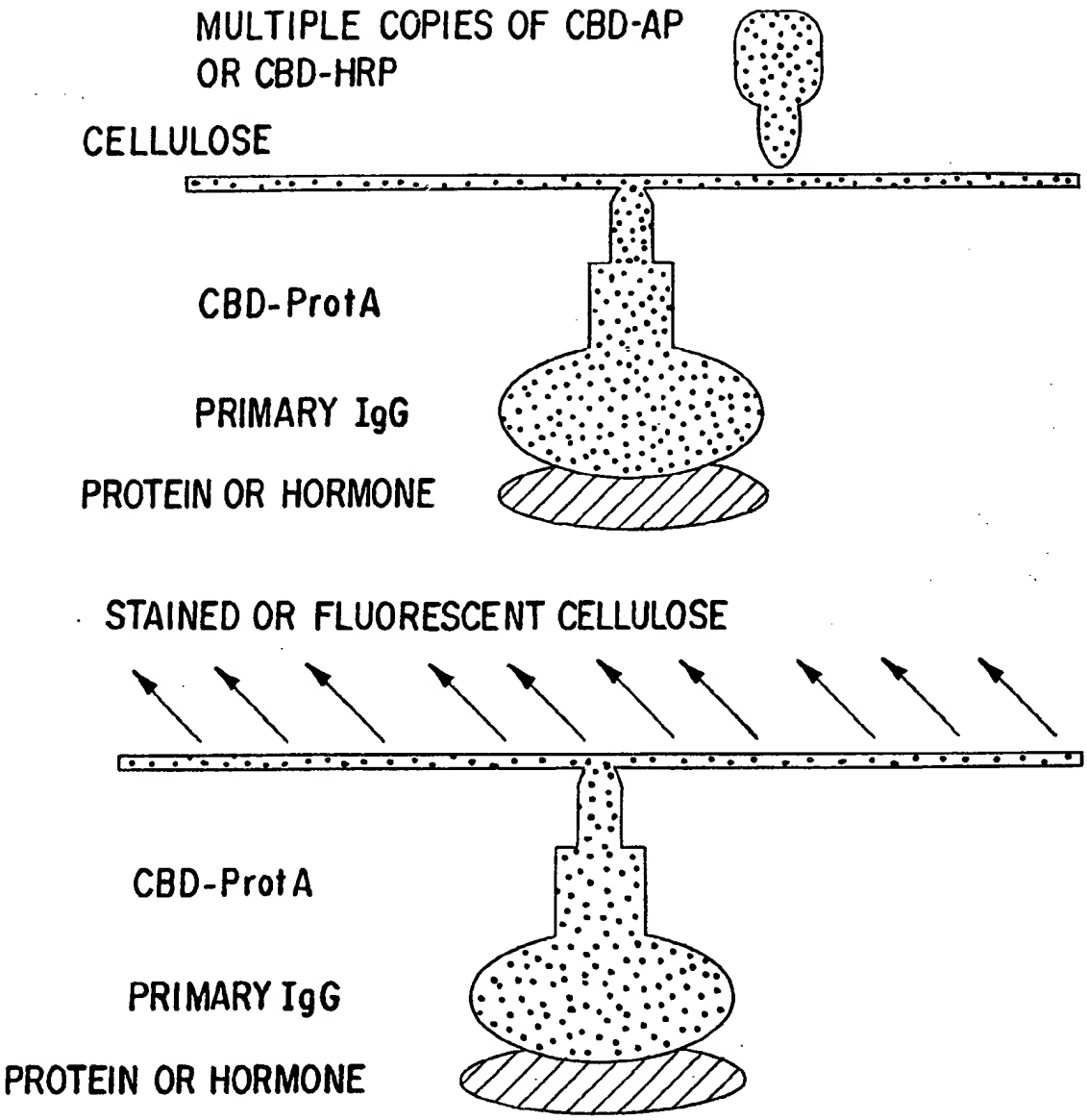
MULTIPLE COPIES OF CBD-AP
OR CBD-HRP
CELLULOSE
CBD-ProtA
PRIMARY IgG
PROTEIN OR HORMONE
STAINED OR FLUORESCENT CELLULOSE
CBD-ProtA
PRIMARY IgG
PROTEIN OR HORMONE

FIG. 12

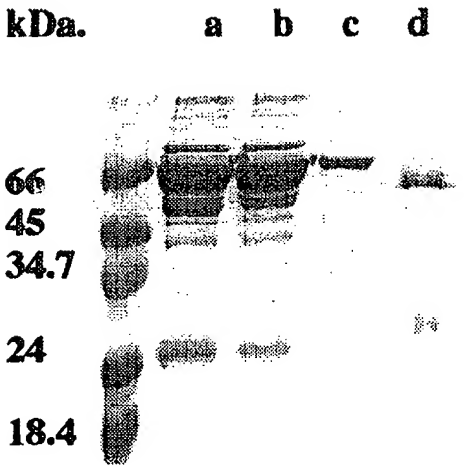

FIG. 13

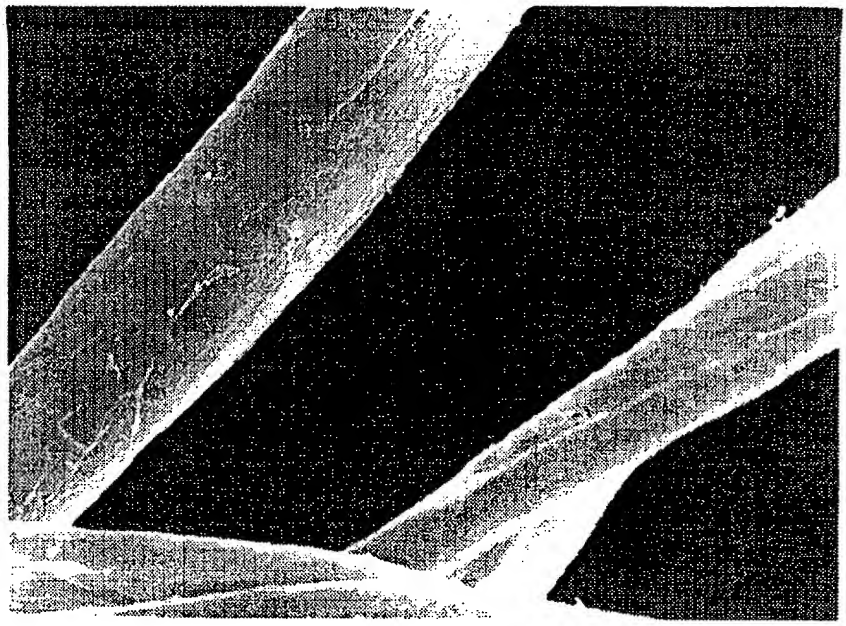

FIG. 14A

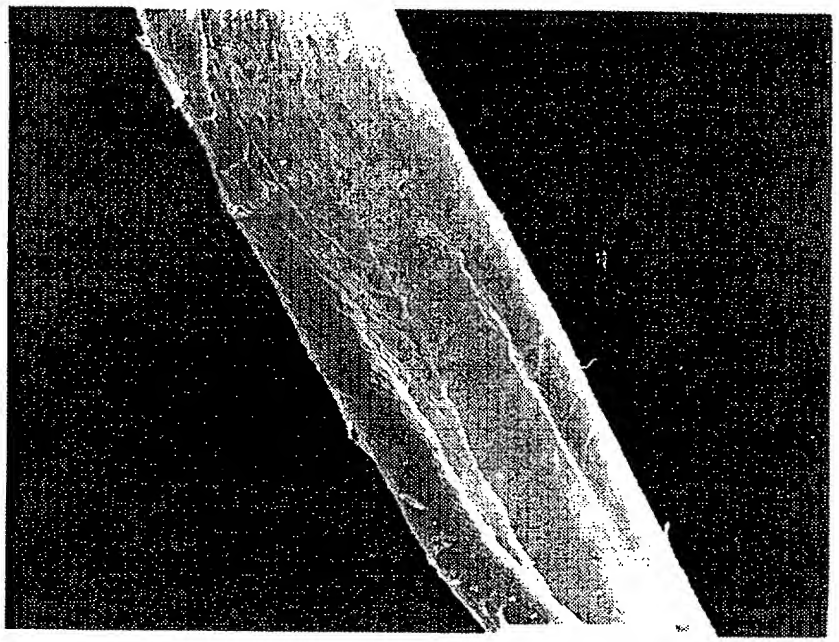

FIG. 14B

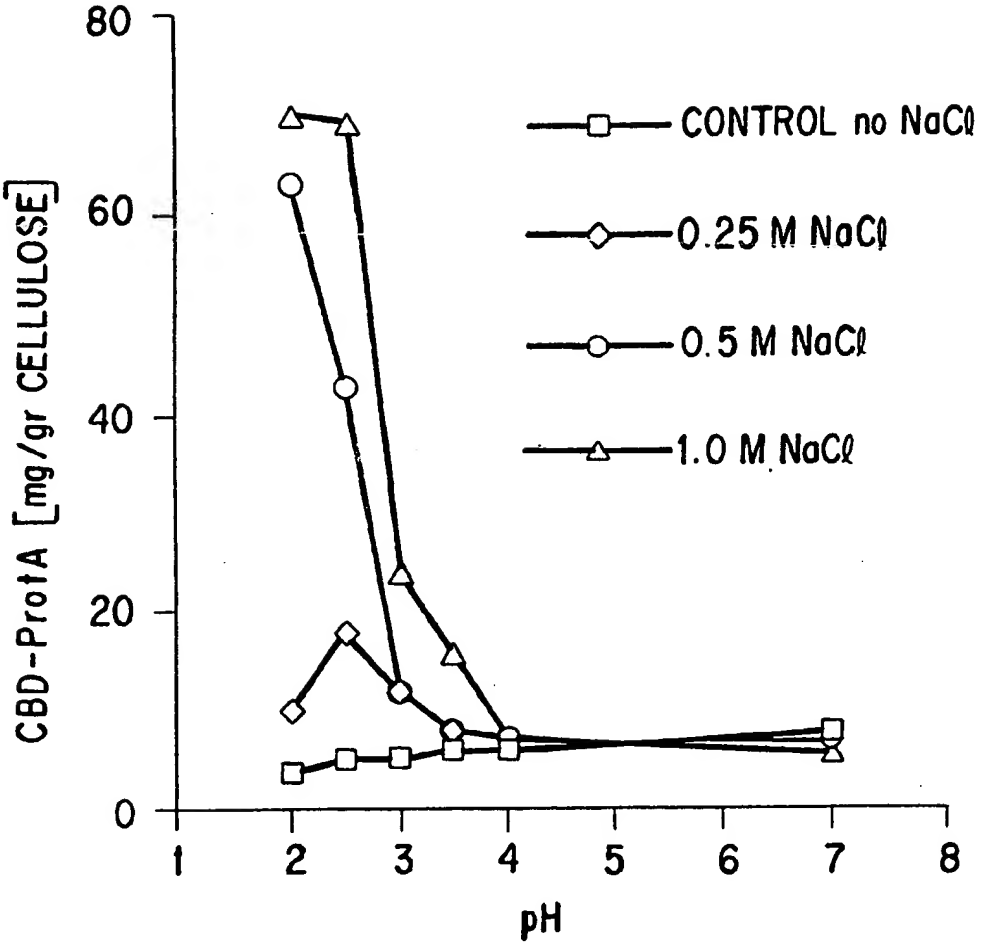
80
60
40
20
0
CBD-ProtA [mg/gr CELLULOSE]
1
2
3
4
5
6
7
8
pH
CONTROL no NaCl
0.25 M NaCl
0.5 M NaCl
1.0 M NaCl

FIG.15

a b c

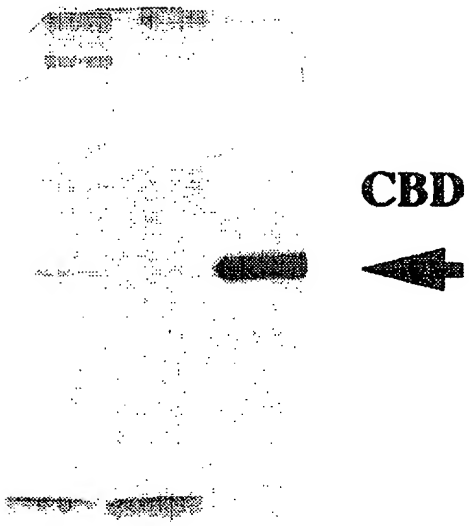

FIG. 16

a b

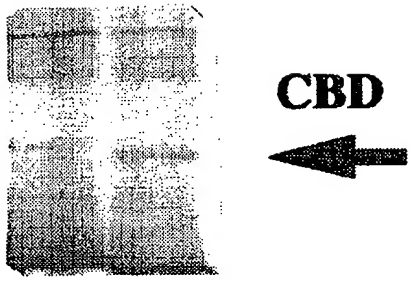

FIG. 17

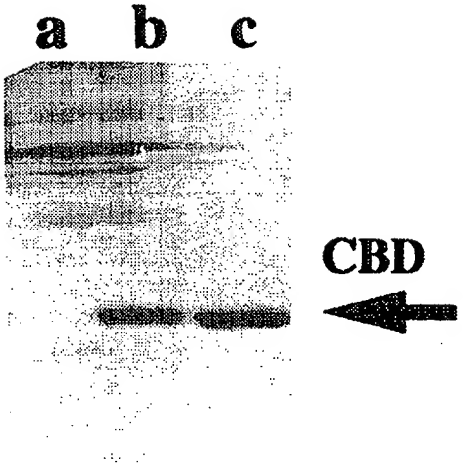

FIG. 18

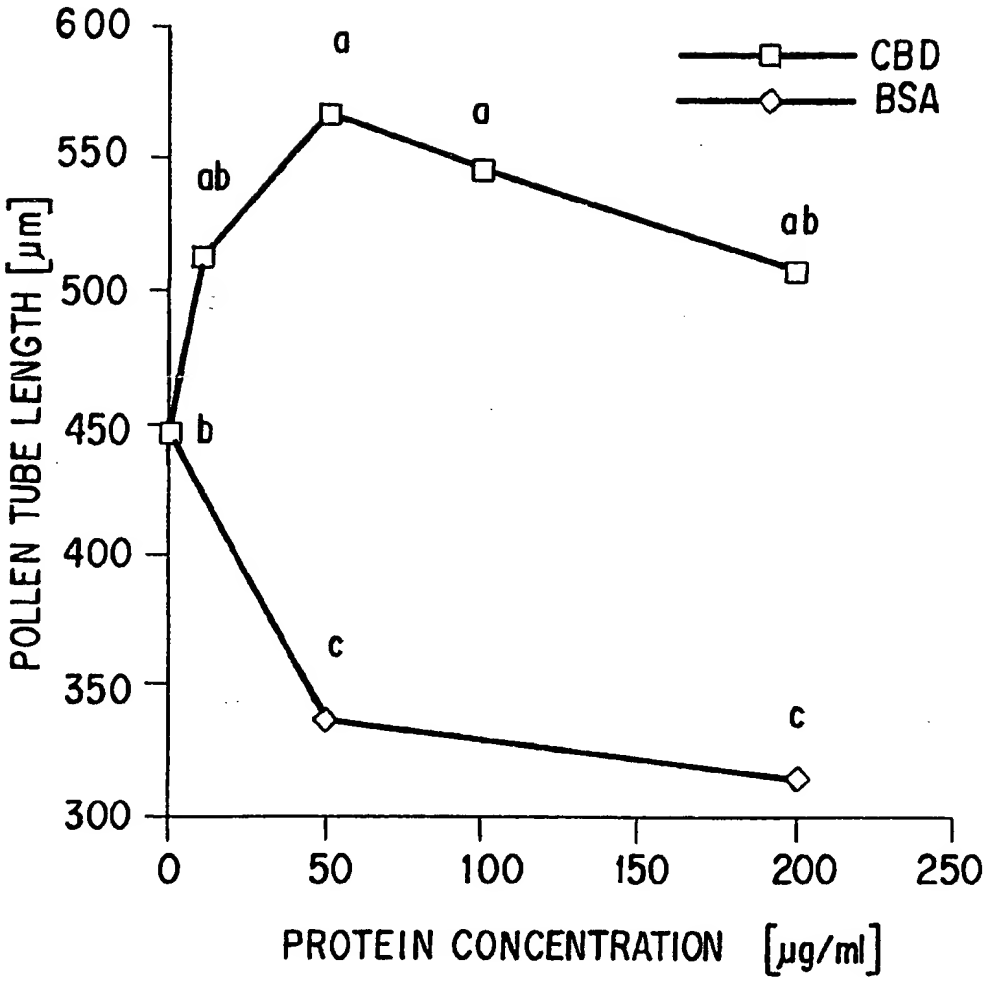
600
550
500
450
400
350
300
POLLEN TUBE LENGTH [µm]
0
50
100
150
200
250
PROTEIN CONCENTRATION [µg/ml]
CBD
BSA
a
a
ab
ab
b
c
c

FIG. 19

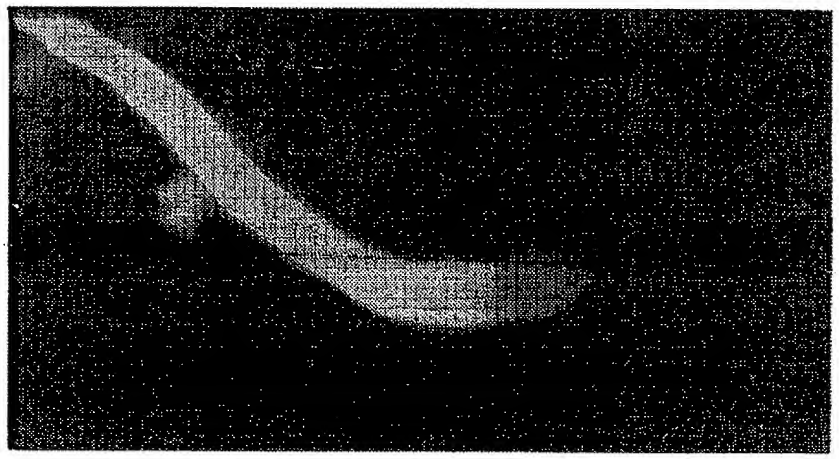

FIG. 20A

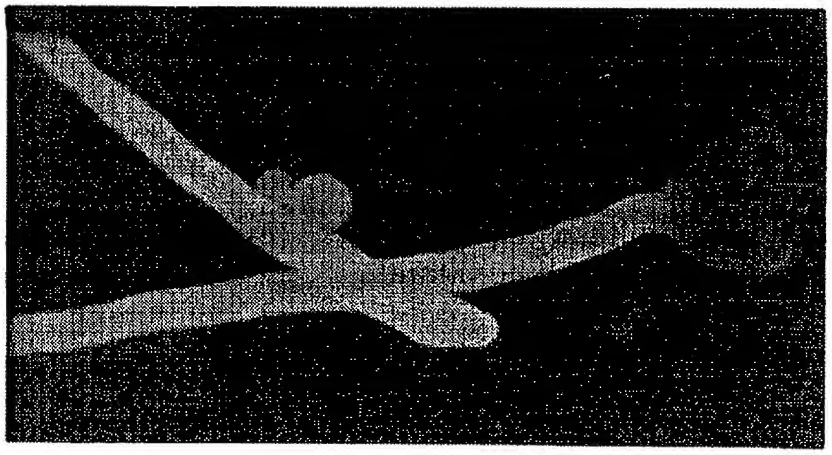

FIG. 20B

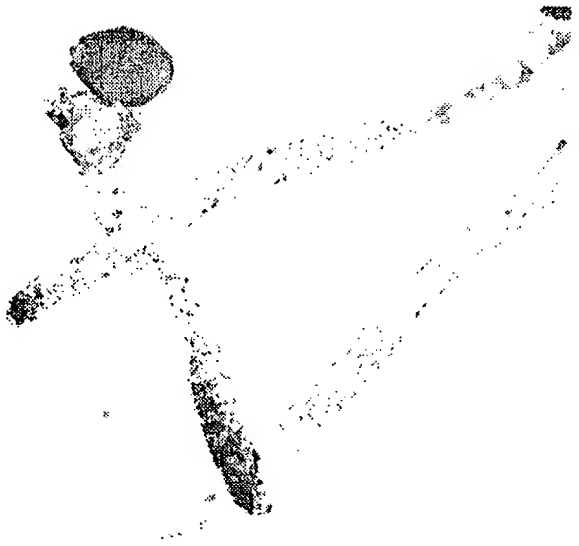

FIG. 21A

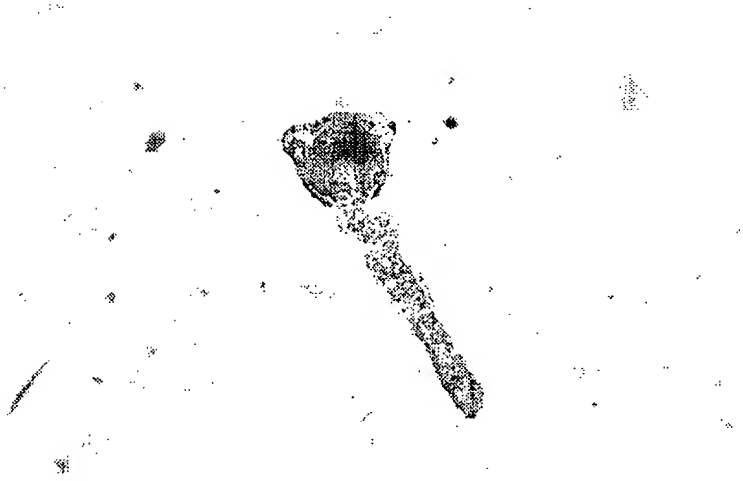

FIG. 21B

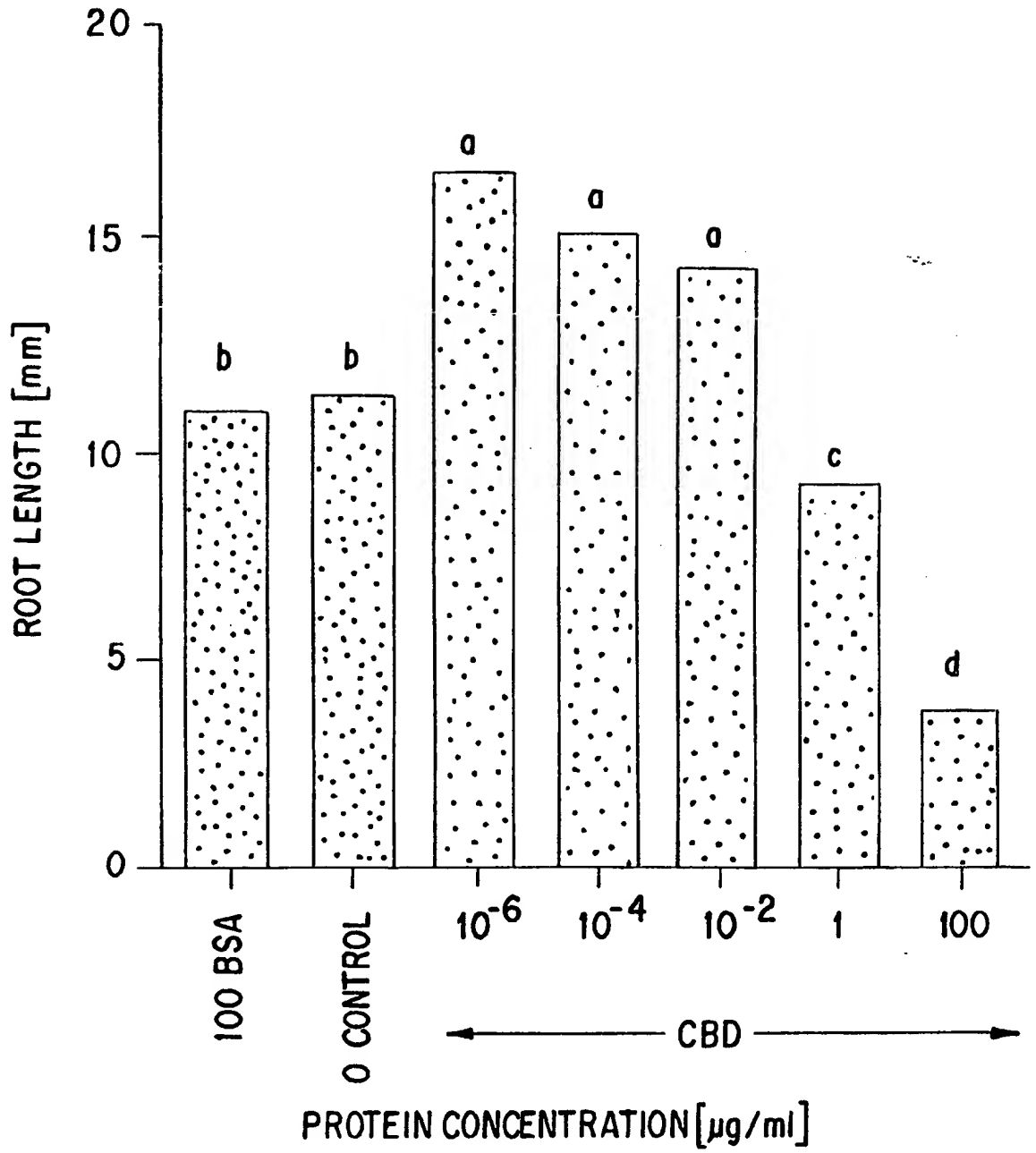
20
15
10
5
0
ROOT LENGTH [mm]
b
b
a
a
a
c
d
100 BSA
0 CONTROL
10-6
10-4
10-2
1
100
CBD
PROTEIN CONCENTRATION [µg/ml]

FIG. 22

FIG. 23

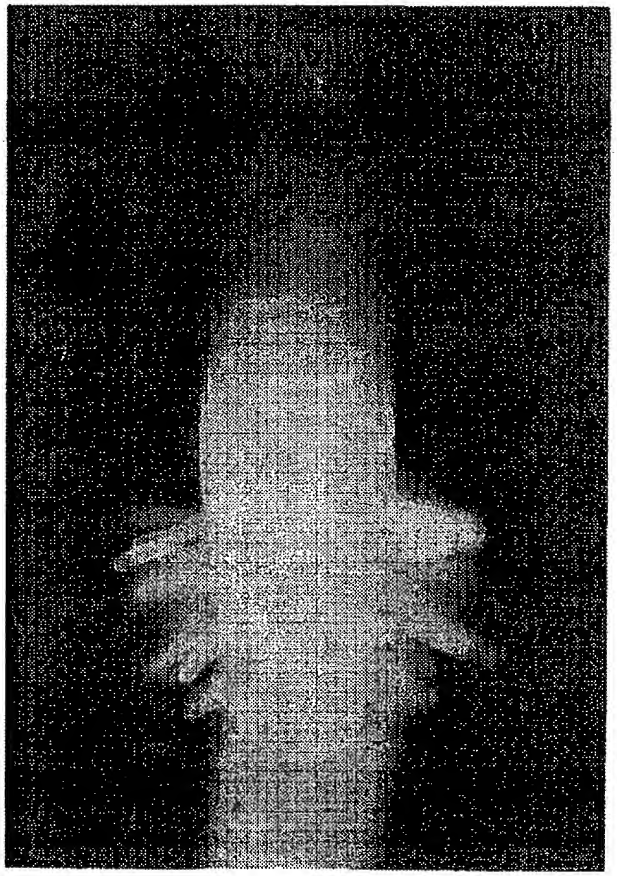

FIG.24

INTERNATIONAL SEARCH REPORT

Int ational application No.
PCT/US94/04132

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.1, 23.4, 23.7, 24.3, 24.33; 435/69.1, 172.1, 252.3, 320.1; 436/501, 512; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; DIALOG; SEQUENCE DATABASES INCLUDING GENBANK AND EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US, A, 5,137,819 (KILBURN ET AL) 11 August 1992, see entire document.	2-9, 11-13, 15-17, 19, 20, 21, 33-37, 39-41, 44, ---- 14, 22-29, 31, 38, 42, 43, 45--87
X ---- Y	FEMS Microbiology Letters, Volume 99, issued 1992, Poole et al, "Identification of the cellulose-binding domain of the cellulosome subunit S1 from Clostridium thermocellum YS", pages 181-186, see entire document.	2, 11-13, 15, 16, 19, 20 ---- 3-9, 14, 17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
14 JULY 1994	22 JUL 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ELIZABETH C. KEMMERER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/US94/04132

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	Molecular and General Genetics, Volume 223, issued 1990, Jauris et al, "Sequence analysis of the Clostridium stercorarium celZ gene encoding a thermoactive cellulase (Avicelase I): Identification of catalytic and cellulose-binding domains", pages 258-267, see entire document.	2, 11-13, 15, 16, 19, 20 ---- 3-9, 11-17, 19, 20
Y, P	US, A, 5,229,501 (KEIFER ET AL) 20 July 1993, see entire document.	14
Y	US, A, 5,122,448 (VAUGHAN ET AL) 16 June 1992, see entire document.	46-77
Y,E	US, A, 5,322,769 (BOLLING ET AL) 21 June 1994, see entire document.	46-77
Y	Biotechnology and Bioengineering, Volume 42, issued 1993, Ong et al, "The cellulose-binding domain (CBDCex) of an exoglucanase from Cellulomonas fimi: production in Escherichia coli and characterization of the polypeptide", pages 401-409, see entire document.	3-9, 11-17, 19, 20
Y	Biochemistry Journal, Volume 273, issued 1991, Durrant et al, "The non-catalytic C-terminal region of endoglucanase E from Clostridium thermocellum contains a cellulose-binding domain", pages 289-293, see entire document.	3-9, 11-17, 19, 20
Y	TIBTECH, Volume 7, issued September 1989, Ong et al, "The cellulose -binding domains of cellulases: tools for biotechnology", pages 239-243, see entire document.	1-89
Y	Biochemistry Journal, Volume 279, issued 1991, Poole et al, "Characterization of hybrid proteins consisting of the catalytic domains of Clostridium and Ruminococcus endoglucanases, fused to Pseudomonas non-catalytic cellulose-binding domains", pages 787-792, see entire document.	22-32
Y	FEBS Letters, Volume 244, Number 1, issued February 1989, Greenwood et al, "Fusion to an endoglucanase allows alkaline phosphatase to bind to cellulose", pages 127-131, see entire document.	22-46

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/US94/04132

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/US94/04132

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C07K 13/00; C07H 17/00; C12P 21/06; C12N 15/00, 1/20; G01N 33/566, 33/563; A61K 37/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/350; 536/23.1, 23.4, 23.7, 24.3, 24.33; 435/69.1, 172.1, 252.3, 320.1; 436/501, 512; 514/2

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-21, 44, and 45, drawn to an isolated cellulose binding domain peptide, DNA encoding same, a PCR kit, plasmids and host cells.
II. Claims 22-36, drawn to a cellulose binding domain fusion protein, plasmids, vectors, host cells, and a method of producing the fusion protein.
III. Claims 37-43, drawn to a method of purifying a cellulose binding domain fusion protein.
IV. Claims 46-70, drawn to a diagnostic kit and immunoassay.
V. Claims 71-77, drawn to a signal amplification system.
VI. Claims 78-87, drawn to a drug delivery system.
Inventions I-VI represent separate inventive concepts. Invention I is directed to the general inventive concept of the recombinant production of a cellulose binding domain peptide, which is useful for the production of antibodies, for example. Invention II is directed to the general inventive concept of the recombinant production of a fusion protein comprising a cellulose binding domain, which is useful for degrading environmental pollutants (as claimed in claim 23), for example. Invention III is directed to the general inventive concept of methods of purifying fusion products comprising the cellulose binding domain, which requires the consideration of purification techniques such as chromatography, which is not required by any of the other inventions. Invention IV is directed to the general inventive concept of diagnostic kits and immunoassays, requiring consideration of binding kinetics and detection sensitivity which is not required by any of the other inventions. Invention V isdirected to the general inventive concept of a signal amplification system, which is useful for in situ hybridization assays. Finally, invention VI is directed to the general inventive concept of a drug delivery system, requiring consideration of disease states, efficacy, toxicity, and conventional drugs, which is not required by any of the other groups. The claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.